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Interactions of ricin with sinusoidal endothelial rat liver cells

Different involvement of two distinct carbohydrate-specific mechanisms in surface binding and internalization

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We have investigated the interactions of the plant toxin ricin with sinusoidal endothelial rat liver cells (EC). In these cells, ricin can be bound and internalized via either cell surface galactosyl residues or mannose receptors. Binding and uptake via galactosyl residues and mannose receptors was studied in the presence of mannan (1 mg/ml) and lactose (50 mM) respectively. Whereas most of the ricin binding was accounted for by cell surface galactosyl residues, uptake of ricin via mannose receptors was much more efficient than uptake via galactosyl residues. Internalized ricin is subject to extensive retroendocytosis (recycling to the cell surface from an early endocytic compartment). Retroendocytosis occurs after internalization of ricin via either pathway and to a much greater extent than for other glycoproteins taken up via mannose receptors of the EC. Hyperosmolarity (150 mM-sucrose), which is known to inhibit endocytosis from coated pits, strongly inhibited ricin uptake via mannose receptors, but had less effect on uptake via galactosyl residues. This suggests that only part of the galactose-specific uptake takes place from coated pits. Protein synthesis in EC was very sensitive to ricin [concn. causing half-maximal inhibition (IC_{50}) = 1.3×10^{-13} M]. Mannan was slightly more effective than lactose in protecting the EC protein synthesis from ricin toxicity.

INTRODUCTION

The plant toxin ricin consists of two polypeptide chains, A and B (M_r approx. 30000 each), linked by a single disulphide bond. The A-chain is a specific *N*-glycosidase which inhibits protein synthesis by removing a single adenine from the 28 S ribosomal RNA (Endo & Tsurugi, 1987; Endo *et al.*, 1987), whereas the B-chain is a lectin which can bind to a variety of galactose-terminated glycoproteins and glycolipids on cell surfaces (Baenziger & Fiete, 1979; Turpin *et al.*, 1984). After binding to cells the ricin molecules are internalized by endocytosis, and subsequently the A-chain is translocated through intracellular membranes into the cytoplasm where it exerts its toxic effect (Olsnes & Sandvig, 1983). The translocation mechanism is not fully understood, and it is not sure whether the whole toxin or only the A-chain is translocated.

Both ricin A- and B-chains are glycosylated and expose terminal mannosyl residues (Foxwell *et al.*, 1987; Kimura *et al.*, 1988). Thus ricin can be subjected to endocytosis mediated by mannose receptors in cells that carry them, in addition to the endocytosis that generally takes place via galactosyl residues (Simmons *et al.*, 1986).

Ricin has been widely used in the preparation of immunotoxins, in which either the entire toxin or the A-chain is conjugated to monoclonal antibodies directed against tumour antigens (Olsnes *et al.*, 1989; Hertler & Frankel, 1989). In order to improve the therapeutic efficiency of immunotoxins, several studies have been devoted to the mechanism of ricin clearance, internalization and intoxication.

Upon intravenous injection of ricin into rats, 30–40% of the injected dose associates with the liver within 30 min (Skilleter *et al.*, 1981; Thorpe *et al.*, 1985; Ramsden *et al.*, 1989). Specificity studies have shown that uptake of ricin in rat liver *in vivo* involves both galactose- and mannose-specific interactions (Thorpe *et al.*, 1985). The parenchymal liver cells, which make up most of the liver tissue in terms of both volume and cell number, bind ricin only via surface galactosyl residues (Skilleter *et al.*, 1981; Decastel *et al.*, 1989), since they do not carry mannose receptors (Kindberg *et al.*, 1990). On the other hand, most of the ricin uptake in the liver is accounted for by mannose-specific endocytosis in the non-parenchymal liver cells (Skilleter *et al.*, 1985). Kupffer cells, the liver macrophages, were initially thought to be responsible for clearance of ricin (Skilleter *et al.*, 1981) and other mannose-terminated ligands (Schlesinger *et al.*, 1978) from the circulation. Kupffer cells are apparently damaged in ricin-induced hepatitis (Bingen *et al.*, 1987), and it was recently claimed that Kupffer cells can be selectively depleted from mouse liver upon injection of ricin (Zenilman *et al.*, 1989). However, the sinusoidal endothelial liver cells (EC), which also carry mannose receptors, are much more efficient than Kupffer cells in removing mannose-terminated glycoconjugates from the circulation *in vivo* (Hubbard *et al.*, 1979; Kindberg *et al.*, 1990). The EC carry a number of different endocytic receptors on their surface and are thought to play a central role in removing several hazardous compounds from the mammalian circulation, such as hydrolytic enzymes and degradation products from connective tissue (Smedsrød *et al.*, 1990). We have previously shown that the mannose receptor of EC mediates extremely rapid endocytosis of mannose-terminated glycoproteins, both *in vivo* and *in vitro* (Magnusson & Berg, 1989; Kindberg *et al.*, 1990). Thus the EC present themselves as a potential candidate for clearing the bulk of intravenously injected ricin from the circulation.

In view of the putative role of the EC in clearance of ricin *in vivo*, it was interesting to characterize the interactions of ricin

Abbreviations used: EC, sinusoidal endothelial rat liver cells; Man-BSA, mannosylated BSA; In/Sur, ratio of internalized to surface-bound ricin; K_e, endocytic rate constant; IC_{50}, concn. causing 50% inhibition.

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with these cells *in vitro*. Here we present results from studies on binding, uptake and retroendocytosis of ricin by isolated EC via mannose receptors and galactosyl residues, and on the inhibition of EC protein synthesis by ricin.

EXPERIMENTAL

Reagents

BSA, collagenase (type I) and yeast mannan were obtained from Sigma. $Na^{125}I$ was obtained from CIS-Oris Industrie (Paris, France) and Nycodenz was from Nycomed A/S (Oslo, Norway). Mannosylated albumin (Man-BSA) was prepared according to Monsigny *et al.* (1984) and radiolabelled according to Pittman *et al.* (1983). All additional chemicals were of analytical grade.

Preparation and radioiodination of ricin

Ricin was prepared from *Ricinus communis* seeds (var. *sanguineus*; Bertrand frères, Angers, France) according to Nicolson & Blaustein (1972). The preparation was homogeneous when tested by PAGE, ultracentrifugation and immunoelectrophoresis. The minimum haemagglutinating dose, tested against human O^- erythrocytes, was 15–17 μg/ml. The ricin concentration was determined by absorbance measurements at 280 nm, using $a = 1.4$ litre $\cdot g^{-1} \cdot cm^{-1}$ (Zentz *et al.*, 1978).

Ricin was iodinated by reacting it (160 μg in 130 μl) with $Na^{125}I$ (59 MBq; 1.6 mCi) in a IodoGen (Pierce)-coated tube. Labelling was performed in 0.1 M-sodium phosphate buffer, pH 7.5, containing 0.15 M-NaCl and 0.15 M-methyl β-D-galactopyranoside in order to protect the galactosyl-binding sites. After 5 min at 20 °C, ^{125}I-ricin was separated from $Na^{125}I$ and methyl β-D-galactopyranoside by gel filtration on a Trisacryl GF 05 column (1 cm × 14 cm; IBF, Paris, France), followed by chromatography on Amberlite IRA-400 (1 cm × 5 cm; Sigma). ^{125}I-Ricin was kept at −20 °C in the presence of 0.25% BSA. Its specific radioactivity was (6–9) × 10^6 c.p.m./μg and the minimum haemaglutinating dose was 18–20 μg/ml. Only 4–6% of the labelled ricin was present as reduced chains, as judged by SDS/PAGE and autoradiography.

Preparation of isolated EC

Isolated EC were prepared as described earlier (Magnusson & Berg, 1989). Briefly, rat livers were perfused with collagenase (Berg & Blomhoff, 1983) and the liver cells were dispersed in incubation buffer (Tolleshaug *et al.*, 1977) containing 1% BSA. A non-parenchymal cell fraction was prepared by differential centrifugation and the EC were isolated by centrifugal elutriation in a Beckman J-6M/E centrifuge equipped with a Beckman JE-5.0 elutriation rotor with a standard separation chamber. The EC were collected at a rotor speed of 2500 rev./min and a flow rate of 22 ml/min and purified further by centrifugation in 20% (w/v) Nycodenz in incubation buffer (1.12 g/ml). EC isolated by this method are essentially 100% viable, as assessed by the Trypan Blue exclusion test (Berg *et al.*, 1972), and were consistently less than 1% contaminated with Kupffer cells, as assessed by staining for endogenous peroxidase activity (Knook & Sleyster, 1977). The yield of elutriated EC was typically (8–12) × 10^6 cells per g of liver (wet weight).

Binding and uptake of ricin by EC

Suspensions of EC in incubation buffer containing 1% BSA were incubated in sealed Erlenmeyer flasks in the presence of ^{125}I-ricin and various concentrations of unlabelled ricin, with or without 50 mM-lactose and 1 mg of mannan/ml. Binding studies were carried out at 4 °C. Aliquots of the incubation mixtures were removed at various time points, the cells were washed three times with incubation buffer containing 1% BSA, and cell-associated radioactivity was measured. Uptake and degradation studies were carried out at 37 °C. Aliquots of the incubation mixtures were layered on top of a mixture of dibutyl phthalate/dinonyl phthalate (3:1) and centrifuged for 30 s at 2000 *g*, and the radioactivity associated with the cell pellets was measured. Aliquots of the supernatants were precipitated with an equal volume of 20% trichloroacetic acid, and both supernatant and pellet were assayed for radioactivity. Uptake was defined as the sum of the cell-associated radioactivity and the increase in acid-soluble radioactivity in the supernatant.

For determination of surface-bound radioactivity during uptake studies, aliquots of the incubation mixture were removed at various time points, the cells were washed three times with cold incubation buffer and then resuspended in ice-cold dissociation buffer (incubation buffer containing 50 mM-lactose and 5 mM-EGTA) for 30 min, and the radioactivity released into the medium was measured. For retroendocytosis studies, the EC were allowed to take up ^{125}I-ricin for 15 min, then were washed three times with ice-cold dissociation buffer containing 1% BSA and incubated further at 37 °C in dissociation buffer. The medium was assayed for acid-precipitable radioactivity at various time points. Samples of the medium after a 30 min incubation at 37 °C were analysed by SDS/PAGE (Laemmli, 1970) and autoradiography.

Cytotoxicity studies

Studies on inhibition of protein synthesis in EC were carried out in the presence or absence of 50 mM-lactose or 1 mg of mannan/ml as previously described (Emmanuel *et al.*, 1988). The cells were incubated at 37 °C; first for 90 min in the presence of various concentrations of ricin, then for 60 min in the absence of ricin, and finally for 90 min in the presence of [^{3}H]leucine (0.37 MBq/ml; 5.18 TBq/mmol; Amersham) in leucine-free minimal essential Eagle's medium (Gibco BRL). At the end of the incubation, triplicate samples were precipitated by addition of an equal volume of 20% trichloroacetic acid, then the precipitate was washed with 10% trichloroacetic acid and dissolved in 0.1 M-NaOH. Radioactivity was measured in a Packard Tri-Carb 2450 liquid scintillation counter, using Insta-Gel (Packard) as the scintillation fluid. Toxicity was determined from the inhibition of [^{3}H]leucine incorporation into acid-precipitable material in ricin-treated cells compared with untreated (control) cells.

Computer fitting of data

Data on binding of ^{125}I-ricin to EC versus time and release of preloaded ^{125}I-ricin from EC versus time (retroendocytosis) were fitted to a first-order rate equation using the Enzfitter data analysis program (Elsevier Science Publishers, Amsterdam, The Netherlands). Data on binding of ^{125}I-ricin to EC versus ricin concentration were fitted to the appropriate binding equations using the same program.

RESULTS

Binding of ricin to isolated EC

Binding of ^{125}I-ricin to isolated EC reached equilibrium within 2 h at 4 °C (Fig. 1). Based on the observed carbohydrate specificity of the binding, it could be separated into two components. At the ricin concentration used (12 nM), addition of

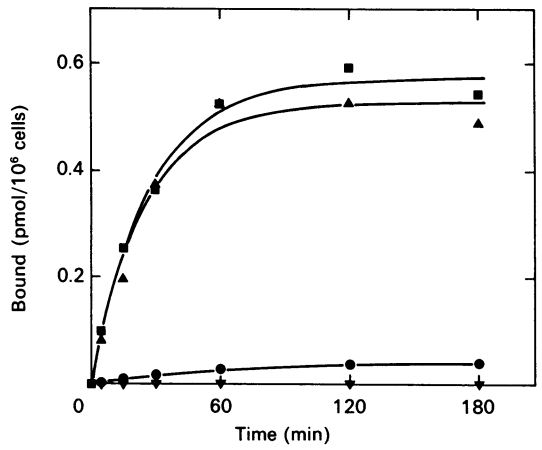

Fig. 1. Binding of ricin to EC as a function of time

EC (1.8×10^6/ml) were incubated with 12 nM-^{125}I-ricin at 4 °C in incubation buffer containing 1% BSA without (■) or with 1 mg of mannan/ml (▲), 50 mM-lactose (●) or both (▼): Samples were removed at each time point and binding was assayed as described in the Experimental section. The lines show fittings to a first-order rate equation (see text).

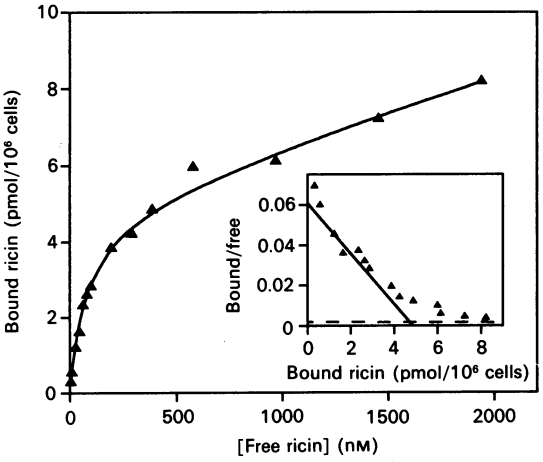

Fig. 2. Binding of ricin to EC galactosyl residues at equilibrium

EC (1.4×10^6/ml) were incubated at 4 °C in incubation buffer containing 1% BSA and 1 mg of mannan/ml with different concentrations of ^{125}I-ricin and unlabelled ricin. Binding was assayed after 2 h as described in the Experimental section. Non-specific binding, measured in the presence of 1 mg of mannan/ml and 50 mM-lactose, has been subtracted. Lines show fittings to a binding equation for two binding sites (see text). The inset shows the corresponding Scatchard plot, with the lines corresponding to the high-affinity (continuous) and low-affinity (broken) binding sites shown. The Figure shows results from a representative experiment.

50 mM-lactose and 1 mg of mannan/ml inhibited binding by 92.9 ± 4.2% and 7.1 ± 0.4% respectively. Addition of both inhibitors together abolished all binding activity. EGTA inhibited binding to a similar extent as mannan, suggesting that these two inhibitors act on a common mannose-specific and Ca^{2+}-dependent binding mechanism. Apparently the lactose-sensitive component (93%) represents binding of ricin B-chain to galactosyl residues at the EC surface, whereas the mannan-sensitive component (7%) represents binding of ricin by mannose receptors at the EC surface, which is Ca^{2+}-dependent (Magnusson & Berg, 1989). After binding to equilibrium at 4 °C, ^{125}I-ricin could

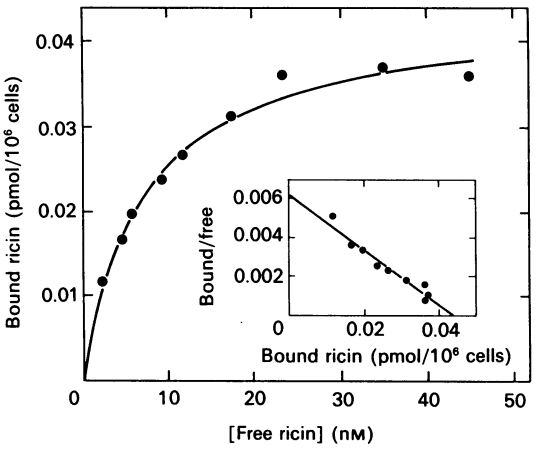

Fig. 3. Binding of ricin to EC mannose receptors at equilibrium

EC (1.1×10^6/ml) were incubated at 4 °C in incubation buffer containing 1% BSA and 50 mM-lactose with different concentrations of ^{125}I-ricin and unlabelled ricin. Binding was assayed after 2 h as described in the Experimental section. Non-specific binding, measured in the presence of 1 mg of mannan/ml and 50 mM-lactose, has been subtracted. The inset shows the corresponding Scatchard plot. Lines show fittings to a binding equation for one binding site (see text). The Figure shows results from a representative experiment.

be rapidly dissociated from both galactosyl residues and mannose receptors by adding 50 mM-lactose and 5 mM-EGTA, respectively (results not shown).

The concentration-dependence of binding of ^{125}I-ricin via each of the two mechanisms was studied in the presence of mannan (1 mg/ml) and 50 mM-lactose respectively. The saturation curve for binding of ricin to galactosyl residues is shown in Fig. 2. The corresponding Scatchard plot (Fig. 2, inset) was biphasic, suggesting the existence of two types of galactosyl-binding sites on the EC, with high and low affinities. Computer fitting of the binding data yielded $(2.9 \pm 0.7) \times 10^6$ high-affinity binding sites and $(4.6 \pm 1.3) \times 10^7$ low-affinity binding sites per cell, with dissociation constants of $(8.0 \pm 0.4) \times 10^{-8}$ M and $(4.0 \pm 1.3) \times 10^{-5}$ M respectively.

The saturation curve and Scatchard plot for binding to mannose receptors yielded a value of $(3.1 \pm 2.1) \times 10^4$ receptors per cell, with a dissociation constant of $(5.3 \pm 1.3) \times 10^{-9}$ M (Fig. 3).

Uptake of ricin by isolated EC

Uptake of ^{125}I-ricin showed a markedly different carbohydrate specificity from the binding (Fig. 4). Whereas most of the binding was lactose-sensitive and only a small fraction was mannan-sensitive, the opposite was observed for the uptake (3 times more taken up via mannose receptors than via galactosyl residues after 30 min; 0.46 and 0.16 pmol/10^6 cells respectively). The uptake was completely blocked in the presence of both inhibitors. Degradation was low compared with the extent of uptake (less than 5% of internalized ricin after 30 min; results not shown).

Rate of endocytosis of ricin by isolated EC

The finding that the extent of binding and uptake via the two different mechanisms was reciprocally arranged indicated that the rate of endocytosis could be one or two orders of magnitude greater for the small fraction bound to mannose receptors than for the large fraction bound to surface galactosyl residues. To investigate this, we measured the endocytotic rate constants for both pathways by constructing In/Sur (ratio of internalized to

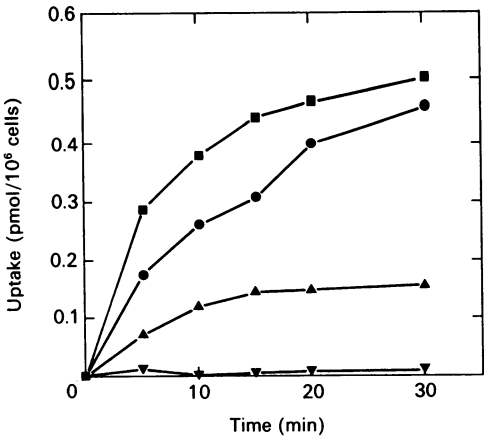

Fig. 4. Uptake of ricin by EC as a function of time

EC (1.0×10^6/ml) were incubated with 12 mM-^{125}I-ricin at 37 °C in incubation buffer containing 1 % BSA without (■) or with 1 mg of mannan/ml (▲), 50 mM-lactose (●) or both (▼). Samples were removed at each time point and uptake was assayed as described in the Experimental section.

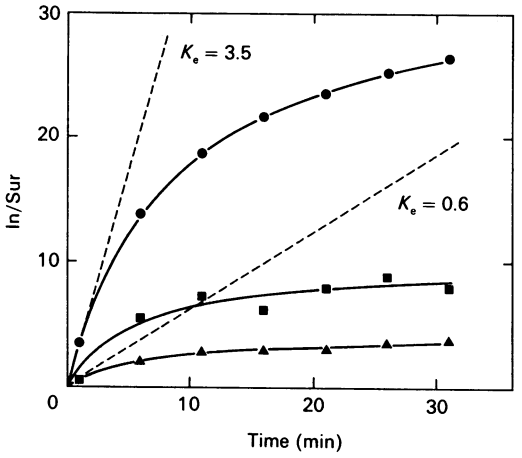

Fig. 5. In/Sur plot for internalization of ricin by EC as a function of time

EC [(7–11) $\times 10^6$/ml] were incubated with 19 nM-^{125}I-ricin at 37 °C in incubation buffer containing 1 % BSA without (■) or with 1 mg of mannan/ml (▲) or 50 mM-lactose (●). Samples were removed at each time point and surface-bound and internalized ricin was assayed as described in the Experimental section. The endocytic rate constants (K_e) were determined from the slopes of the initial part of the curves (broken lines).

surface-bound ricin) plots for the uptake (Wiley & Cunningham, 1982). Instead of yielding straight lines, as expected for simple vectorial internalization, the In/Sur plots were clearly curved (Fig. 5). However, the initial rate of endocytosis via mannose receptors estimated from the initial part of the curve was much higher than the rate of endocytosis via galactosyl residues (3.5 min^{-1} and 0.6 min^{-1} respectively). As expected, the rate of uptake estimated in the absence of inhibitors was between these values (1.5 min^{-1}).

Retroendocytosis of ricin from isolated EC

A possible explanation of the observed curvature of the In/Sur plots could be extensive retroendocytosis (recycling of internalized ligand from an early endocytotic compartment to the cell surface), which we have previously observed in these cells

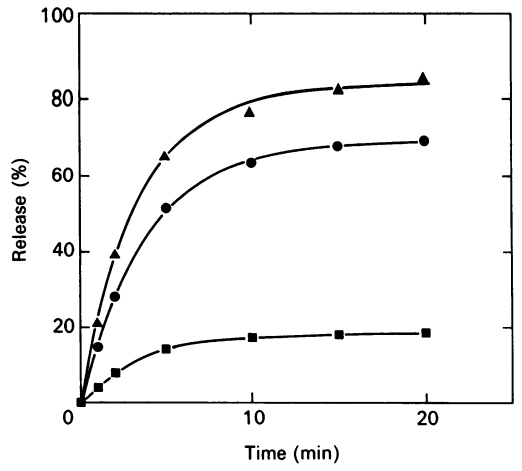

Fig. 6. Retroendocytosis of ricin and Man-BSA from EC

EC (1.9×10^6/ml) were incubated for 15 min at 37 °C in incubation buffer containing 1 % BSA with 19 nM-^{125}I-ricin in the presence of 1 mg of mannan/ml (▲) or 50 mM-lactose (●), or with 10 nM-^{125}I-Man-BSA (■). The cells were washed with cold dissociation buffer containing 1 % BSA, and then incubated further at 37 °C in dissociation buffer. Samples were removed at each time point and acid-precipitable radioactivity in the medium was measured. Results are expressed as percentages of radioactivity associated with the cells at the beginning of the last incubation period. The lines show fittings to a first-order rate equation (see text).

(Magnusson & Berg, 1989). When retroendocytosis occurs, the absolute rate of internalization can only be observed initially, before any recycled ligand reaches the cell surface. After that, the apparent (net) rate of internalization is lower than the absolute rate because of the recycling. Indeed, internalized ricin is released from the cells to a much larger extent than Man-BSA and other ligands taken up by mannose receptors in these cells (Magnusson & Berg, 1989) (Fig. 6). Maximal release of ricin from the cells was greater if it was internalized via galactosyl residues (83.0 ± 0.9 %) than via mannose receptors (69.5 ± 0.1 %), while maximal release of Man-BSA was only 18.3 ± 0.1 %. However, the rate constant for the release was similar for all three curves (0.3 min^{-1}). The released ricin was unchanged in M_r and the interchain disulphide bond was intact, as judged by SDS/PAGE and autoradiography (results not shown).

Endocytosis from coated or uncoated pits

Although most endocytic receptors are internalized from coated pits, endocytosis can also take place outside coated pits (Sandvig *et al.*, 1987; Hubbard, 1989). Besides endocytosis from coated pits (van Deurs *et al.*, 1985), it has been claimed that, in several cell lines, ricin is mainly internalized outside coated pits (Moya *et al.*, 1985; Sandvig *et al.*, 1987; Sandvig & van Deurs, 1990). However, these cell lines only internalize ricin via surface galactosyl residues, since they do not carry mannose receptors. While mannose receptors on EC are localized in coated pits (Kempka & Kolb-Bachofen, 1988), galactosyl residues are found as constituents of numerous plasma membrane glycoproteins and glycolipids, and are evenly distributed over the cell surface (both inside and outside coated pits). We investigated whether the different rates at which ricin is internalized, when bound to galactosyl residues and mannose receptors respectively, could be related to such different localization of the two categories of binding sites.

Hyperosmotic medium has been reported to selectively inhibit endocytosis from coated pits, leaving endocytosis from uncoated pits intact (Daukas & Zigmond, 1985; Oka *et al.*, 1989). Indeed,

Table 1. Effect of hyperosmotic medium on binding and uptake of ^{125}I-ricin in EC with or without 50 mM-lactose and mannan (1 mg/ml)

EC (3.7×10^6/ml) were incubated with 12 nM-^{125}I-ricin at 4 °C (binding) or 37 °C (uptake) in incubation buffer containing 1 % BSA with or without mannan (1 mg/ml) and 50 mM-lactose. Each incubation was performed in duplicate, with 150 mM-sucrose added to one of each pair. Samples were removed after 120 min (binding) and 30 min (uptake), and cell-associated radioactivity was measured as described in the Experimental section. The Table shows the decrease in binding and uptake caused by 150 mM-sucrose.

	Decrease on addition of 150 mM-sucrose (%)	
Inhibitor added	Binding	Uptake
None (control)	7.1±1.6	63.7±0.6
Lactose (50 mM)	−19.3±4.8	78.0±1.3
Mannan (1 mg/ml)	12.8±7.5	45.3±2.3

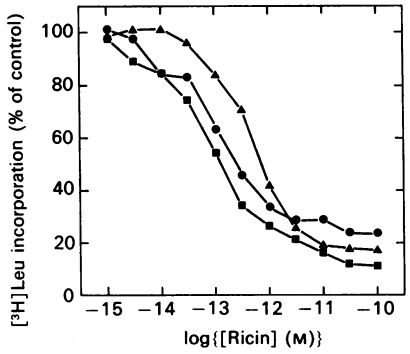

Fig. 7. Inhibition of EC protein synthesis by ricin

EC (3.0×10^6/ml) were incubated for 90 min at 37 °C in incubation buffer containing 1 % BSA with various ricin concentrations without (■) or with 1 mg of mannan/ml (▲) or 50 mM-lactose (●). After 60 min of further incubation in the absence of ricin, the cells were transferred to leucine-free minimal essential medium with [^{3}H]leucine (0.37 MBq/ml) and incubated for 90 min at 37 °C. [^{3}H]Leucine incorporation into acid-precipitable proteins in treated cells is expressed as a percentage of incorporation in untreated (control) cells.

hyperosmotic medium (150 mM-sucrose in incubation buffer) decreased uptake of ricin via mannose receptors strongly, while uptake via galactosyl residues was much less decreased (Table 1). On the other hand, 150 mM-sucrose did not lower significantly binding of ricin to the cells via either mechanism. Binding of ricin to mannose receptors was even slightly increased in the presence of 150 mM-sucrose.

Inhibition of EC protein synthesis by ricin

EC protein synthesis was measured after incubation with various concentrations of ricin, with or without 50 mM-lactose and mannan (1 mg/ml) added (Fig. 7). When no inhibitor was added, an IC_{50} value (concn. causing 50% inhibition) of 1.3×10^{-13} M was obtained. In the presence of lactose and mannan, the IC_{50} values were 2.3×10^{-13} and 7.1×10^{-13} M respectively. In other words, a 3-fold higher concentration was required to obtain 50% inhibition of protein synthesis when ricin was taken up via galactosyl residues only than when uptake took place via mannose receptors only.

DISCUSSION

In the present work, we have investigated the mechanisms of binding and internalization of ^{125}I-ricin by rat liver sinusoidal endothelial cells *in vitro*. We found that, although ricin is mainly bound to the cells via surface galactosyl residues, most of the internalized ricin is taken up via mannose receptors.

Biphasic Scatchard curves are generally obtained for binding of ricin to cell surface galactosyl residues. This could be due to either the existence of two types of binding sites on the cells with high and low affinity respectively, or the two galactosyl-binding sites found on the ricin B-chains. However, linear Scatchard plots have been obtained for binding of ricin to surface galactosyl residues on some cell types (hepatocytes; Decastel *et al.*, 1989), and it is therefore generally believed that the two different types of binding sites, indicated by the biphasic Scatchard plot, are of cellular origin. The number of sites and the dissociation constants were of the same order of magnitude as values obtained with various other cell types (Sandvig *et al.*, 1976, 1978; van Deurs *et al.*, 1985; Decastel *et al.*, 1989). The number of mannose receptors ($n_T = 3.1 \times 10^4$ per cell) is much lower than the number of galactosyl-binding sites, and in good agreement with the value obtained from studies of binding of ovalbumin to EC mannose receptors (Magnusson & Berg, 1989). However, the dissociation constant for ricin is ten times lower than the one measured for ovalbumin. This difference in affinity of the two glycoproteins for the mannose receptors may be related to the number and configuration of their terminal mannosyl residues, which are thought to affect the affinity of ligands for mannose receptors (Hoppe & Lee, 1983). Ricin contains several mannose-terminated oligosaccharide chains (Foxwell *et al.*, 1987; Kimura *et al.*, 1988), whereas ovalbumin contains one heterogeneous oligosaccharide chain (Yamashita *et al.*, 1978, 1983).

As previously observed in rat bone marrow macrophages, the number of galactosyl-binding sites on the EC was much higher than the number of mannose receptors (Simmons *et al.*, 1986). However, while galactose-specific uptake accounted for most of the total ricin uptake in the macrophages, uptake via mannose receptors is much more effective in the EC than uptake via surface galactosyl residues. The endocytic rate constant for uptake via mannose receptors ($K_e = 3.5$ min^{-1}), estimated from the initial part of the In/Sur plot, was approx. 6 times higher than the rate constant for uptake via galactosyl residues ($K_e = 0.6$ min^{-1}) and similar to the rate constant obtained for uptake of ovalbumin via EC mannose receptors ($K_e = 4.1$ min^{-1}; Magnusson & Berg, 1989). This difference is of the same order of magnitude as the difference in uptake after 30 min (3-fold).

The observation of a rapid uptake of ricin via mannose receptors in EC *in vitro* may have consequences for the prevailing idea that the Kupffer cells are responsible for most of the ricin uptake in rat liver *in vivo*. Considering that most of the ricin uptake in the liver is a mannose-sensitive uptake in non-parenchymal cells (Thorpe *et al.*, 1985; J. P. Frénoy *et al.*, unpublished work) and that the EC are more efficient than Kupffer cells in the internalization of mannosyl-terminated ligands *in vivo* (Hubbard *et al.*, 1979), we suggest that the main part of ricin uptake in rat liver *in vivo* occurs through the mannose receptors of the EC.

Retroendocytosis of ricin has previously been reported in experimental conditions similar to ours, and ranged from 20–40% within 20 min, depending on the cell line (Sandvig & Olsnes, 1979; McIntosh *et al.*, 1990; Sandvig & van Deurs, 1990). In the present work, we observed a very extensive (> 80%) and fast release of apparently intact ricin molecules from the EC, which may explain the observed curvature of the In/Sur plots. Retroendocytosis of Man-BSA (Fig. 6) and ovalbumin

(Magnusson & Berg, 1989), other ligands for the mannose receptors, is considerably less extensive (8–18%) than ricin retroendocytosis. Ricin is functionally different from the other ligands, since it can also bind to membranes via surface galactosyl residues, and differences in association and dissociation kinetics between the two mechanisms of ricin binding, especially at endosomal pH, may be of importance for the extent of retroendocytosis.

Hyperosmotic sucrose did not inhibit binding of ricin to EC via either pathway markedly, and even stimulated binding of ricin to mannose receptors in a reproducible manner. Interestingly, it has been reported that binding of Man-BSA to macrophage mannose receptors is stimulated by millimolar concentrations of saccharides (Hoppe & Lee, 1982), but the reason for this phenomenon remains obscure. While not inhibiting the binding, hyperosmotic medium decreased uptake of ricin via mannose receptors by 78%, suggesting that the uptake via mannose receptors mainly takes place from coated pits. This is in agreement with electron-microscopic data showing that binding of mannosylated particles to EC occurs exclusively in coated pits, which account for only a small fraction (< 5%) of the cell surface (Kempka & Kolb-Bachofen, 1988). On the other hand, uptake of ricin via galactosyl residues was much less decreased in hyperosmotic medium than was uptake via mannose receptors, indicating that a large fraction of the galactose-specific uptake is independent of coated pits. However, assuming that the fraction of galactose-specific binding that takes place in coated pits is proportional to the fraction of the total cell surface area occupied by coated pits (< 5%), the galactose-specific uptake was decreased to a greater extent in hyperosmotic medium than this fraction would indicate (45%). This could mean either that ricin bound to galactosyl residues is to some extent concentrated in coated pits, or that the fraction of ricin that binds to galactosyl residues in coated pits is internalized more effectively than that which is bound outside coated pits.

We found that EC were very sensitive to protein synthesis inhibition by ricin ($IC_{50} = 1.3 \times 10^{-13}$ M). In similar or identical experimental conditions, IC_{50} values obtained for various cell types ranged from 2×10^{-10} to 5×10^{-9} M (Sandvig *et al.*, 1978; Sandvig & Olsnes 1982; van Deurs *et al.*, 1986; Emmanuel *et al.*, 1988; Decastel *et al.*, 1989). IC_{50} values in the 10^{-13} M range have been obtained previously with only few cell types: non-parenchymal liver cells (Skilleter *et al.*, 1981), rat bone marrow macrophages (Simmons *et al.*, 1986) and Bewo cells (Braham *et al.*, 1988). However, in each case, the total incubation time at 37 °C was greater than 22 h, increasing the apparent cell sensitivity (4 h in our conditions).

Protein synthesis inhibition in the presence of inhibitors showed that endocytosis through both pathways leads to intoxication. Similar results have been reported previously for rat bone marrow macrophages. The concentration of ricin required to inhibit protein synthesis in the macrophages by 50% was identical for both pathways of endocytosis, but in these cells the galactose-specific uptake accounted for a much larger fraction of the total ricin uptake than uptake via mannose receptors (Simmons *et al.*, 1986). In comparison, a 3× higher ricin concentration was needed to inhibit protein synthesis in EC by 50% when ricin was taken up via galactosyl residues only than when uptake took place via mannose receptors only, which probably reflects the finding that, in the EC, uptake of ricin via mannose receptors is 3–6-fold more effective than uptake via galactosyl residues.

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REFERENCES

Baenziger, J. & Fiete, D. (1979) J. Biol. Chem. **254**, 9795–9799
Berg, T. & Blomhoff, R. (1983) in Iodinated Density Gradient Media: A Practical Approach (Rickwood, D., ed.), pp. 173–174, IRL Press, Oxford
Berg, T., Boman, D. & Seglen, P. O. (1972) Exp. Cell Res. **72**, 571–574
Bingen, A., Creppy, E. A., Gut, J. P., Dirheimer, G. & Kirn, A. (1987) J. Submicrosc. Cytol. **19**, 247–256
Braham, K., Junqua, S., Tursz, T., Le Pecq, J. B. & Lipinski, M. (1988) Cancer Res. **48**, 806–811
Daukas, G. & Zigmond, S. H. (1985) J. Cell Biol. **101**, 1673–1679
Decastel, M., Haentjens, G., Aubéry, M. & Goussault, Y. (1989) Exp. Cell Res. **180**, 399–408
Emmanuel, F., Turpin, E., Alfsen, A. & Frénoy, J. P. (1988) Anal. Biochem. **173**, 134–141
Endo, Y. & Tsurugi, K. (1987) J. Biol. Chem. **262**, 8128–8130
Endo, Y., Mitsui, K., Motizuki, M. & Tsurugi, K. (1987) J. Biol. Chem. **262**, 5908–5912
Foxwell, B. M. J., Blakey, D. C., Brown, A. N. F., Donovan, T. A. & Thorpe, P. E. (1987) Biochim. Biophys. Acta **923**, 59–65
Hertler, A. A. & Frankel, A. E. (1989) J. Clin. Oncol. **7**, 1932–1942
Hoppe, C. A. & Lee, Y. C. (1982) J. Biol. Chem. **257**, 12831–12834
Hoppe, C. A. & Lee, Y. C. (1983) J. Biol. Chem. **258**, 14193–14199
Hubbard, A. L. (1989) Curr. Opin. Cell Biol. **1**, 675–683
Hubbard, A. L., Wilson, G., Ashwell, G. & Stukenbrok, M. (1979) J. Biol. Chem. **83**, 47–64
Kempka, G. & Kolb-Bachofen, V. (1988) Exp. Cell Res. **176**, 38–48
Kimura, Y., Hase, S., Kobayashi, Y., Kyogoku, Y., Ikenaka, T. & Funatsu, G. (1988) J. Biochem. (Tokyo) **103**, 944–949
Kindberg, G. M., Magnusson, S., Berg, T. & Smedsrød, B. (1990) Biochem. J. **270**, 197–203
Knook, D. L. & Sleyster, E. C. (1977) in Kupffer Cells and Other Liver Sinusoidal Cells (Wisse, E. & Knook, D. L., eds.), pp. 273–288, Elsevier/North-Holland Biomedical Press, Amsterdam
Laemmli, U. K. (1970) Nature (London) **227**, 680–685
Magnusson, S. & Berg, T. (1989) Biochem. J. **257**, 651–656
McIntosh, D. P., Timar, J. & Davies, A. J. S. (1990) Eur. J. Cell Biol. **52**, 77–86
Monsigny, M., Roche, A. C. & Midoux, P. (1984) Biol. Cell **51**, 187–196
Moya, M., Dautry-Varsat, A., Goud, B., Louvrard, D. & Boquet, P. (1985) J. Cell Biol. **101**, 548–559
Nicolson, G. L. & Blaustein, J. (1972) Biochim. Biophys. Acta **266**, 543–547
Oka, J. A., Christensen, M. D. & Weigel, P. H. (1989) J. Biol. Chem. **264**, 12016–12024
Olsnes, S. & Sandvig, K. (1983) in Receptor Mediated Endocytosis (Cuatrecasas, P. & Roth, T. F., eds.), pp. 188–236, Chapman and Hall, London
Olsnes, S., Sandvig, K. & Petersen, O. W. (1989) Immunol. Today **10**, 291–295
Pittman, R. C., Carew, T. E., Glass, C. K., Green, S. R., Taylor, C. A. & Attie, A. D. (1983) Biochem. J. **212**, 791–800
Ramsden, C. S., Drayson, M. T. & Bell, E. B. (1989) Toxicology **55**, 161–171
Sandvig, K. & Olsnes, S. (1979) Exp. Cell Res. **121**, 15–25
Sandvig, K. & Olsnes, S. (1982) J. Biol. Chem. **257**, 7504–7513
Sandvig, K. & van Deurs, B. (1990) J. Biol. Chem. **265**, 6382–6388
Sandvig, K., Olsnes, S. & Pihl, A. (1976) J. Biol. Chem. **251**, 3877–3988
Sandvig, K., Olsnes, S. & Pihl, A. (1978) Eur. J. Biochem. **82**, 13–23
Sandvig, K., Olsnes, S., Petersen, O. W. & van Deurs, B. (1987) J. Cell Biol. **105**, 679–689
Schlesinger, P. H., Doebber, T. W., Mandell, B. F., White, R., DeSchryver, C., Rodman, J. S., Miller, M. J. & Stahl, P. D. (1978) Biochem. J. **176**, 103–109
Simmons, B., Stahl, P. D. & Russell, J. H. (1986) J. Biol. Chem. **261**, 7912–7920
Skilleter, D. N., Paine, A. J. & Stirpe, F. (1981) Biochim. Biophys. Acta **677**, 495–500
Skilleter, D. N., Price, R. J. & Thorpe, P. E. (1985) Biochim. Biophys. Acta **842**, 12–21

Smedsrød, B., Pertoft, H., Gustafson, S. & Laurent, T. C. (1990) Biochem. J. **266**, 313–327
Thorpe, P. E., Detre, S. I., Foxwell, B. M. J., Brown, A. N. F., Skilleter, D. N., Wilson, G., Forrester, J. A. & Stirpe, F. (1985) Eur. J. Biochem. **147**, 197–206
Tolleshaug, H., Berg, T., Nilsson, M. & Norum, K. R. (1977) Biochim. Biophys. Acta **499**, 73–84
Turpin, E., Goussault, Y., Lis, H. & Sharon, N. (1984) Exp. Cell Res. **152**, 486–492
van Deurs, B., Ryde Pedersen, L., Sundan, A., Olsnes, S. & Sandvig, K. (1985) Exp. Cell Res. **159**, 679–689
van Deurs, B., Tønnessen, T. I., Petersen, O. W., Sandvig, K. & Olsnes, S. (1986) J. Cell Biol. **102**, 37–47
Wiley, H. S. & Cunningham, D. D. (1982) J. Biol. Chem. **257**, 4222–4229
Yamashita, K., Tachibana, Y. & Kobata, A. (1978) J. Biol. Chem. **253**, 3862–3869
Yamashita, K., Ueda, I. & Kobata, A. (1983) J. Biol. Chem. **258**, 14144–14147
Zenilman, M. E., Fiani, M., Stahl, P. D., Brunt, E. M. & Flye, M. W. (1989) Transplantation **47**, 200–203
Zentz, C., Frénoy, J. P. & Bourrillon, R. (1978) Biochim. Biophys. Acta **536**, 18–26

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