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Endocytosis of ricin by rat liver cells *in vivo* and *in vitro* is mainly mediated by mannose receptors on sinusoidal endothelial cells

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Upon intravenous injection into rats, the plant toxin ricin was rapidly cleared from the circulation by the liver. Among the different liver cell populations, most of the injected ricin associated with the sinusoidal endothelial cells (EC), whereas the liver parenchymal cells (PC) and Kupffer cells (KC) yielded minor contributions to the total liver uptake *in vivo*. Co-injection of mannan strongly inhibited ricin uptake by the EC, showing that it was mediated by mannose receptors. On the other hand, co-injection of lactose, which inhibits the galactose-specific association of ricin with cells, enhanced ricin uptake by the EC. The carbohydrate-dependency of the EC contribution to the uptake of ricin *in vivo* was reflected in the carbohydrate-dependency of the uptake *in vivo* by whole liver. *In vitro*, the EC also endocytosed ricin more efficiently than did the PC or KC. Whereas uptake *in vitro* in the EC was mainly mannose-specific, uptake in the two other cell types was mainly galactose-specific. Western blotting showed that the mannose receptors of liver non-parenchymal cells are identical with the mannose receptor previously isolated from alveolar macrophages. The mannose receptors are expressed at a higher level in EC than in KC. Ligand blotting showed that, in the presence of lactose, the mannose receptor is the only protein in the EC that binds ricin, and the binding is mannose-specific and Ca^{2+}-dependent.

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 toxicity of ricin resides in the A-chain, which is a N-glycosidase that specifically removes a single adenine residue from 28 S rRNA, thereby inactivating the ribosomes and inhibiting protein synthesis (Endo and Tsurugi, 1987). The B-chain is a galactose-specific lectin that mediates binding of the toxin to cells through a variety of galactose-terminated glycoproteins and glycolipids on cell surfaces (Baenziger and Fiete, 1979). Moreover, both ricin A- and B-chains are glycosylated and expose terminal mannosyl residues (Foxwell et al., 1985). Thus, in addition to the binding to galactosyl residues, ricin can bind to endocytic mannose receptors on cells that carry those, i.e. macrophages (Simmons et al., 1986) and liver endothelial cells (Magnusson et al., 1991). Upon binding to cells by either mechanism, ricin is internalized by endocytosis and subsequently the A-chain is translocated through the membranes of intracellular compartments into the cytoplasm, where it exerts its toxic effect (Olsnes and Sandvig, 1983). Neither the mechanism nor the intracellular site of the translocation are known, but it has been suggested that ricin must be transported from the endocytic pathway to the exocytic pathway (i.e. the *trans*-Golgi network) for translocation to occur (Sandvig et al., 1986; Youle and Colombatti, 1987; van Deurs et al., 1987).

Ricin has been widely used in attempts to prepare therapeutic immunotoxins, by conjugating either the entire toxin or the A-chain to monoclonal antibodies directed against specific target cells, e.g. tumour cells (reviewed by Press, 1991). Several studies have therefore addressed the pharmacokinetics, biodistribution and toxicity of ricin and ricin-based immunotoxins in mammalian species. Upon intravenous administration, ricin may bind to blood cells, vascular endothelial cells or any other cell type adjacent to the circulation, by virtue of the lectin activity of the B-chain. Consequently, for construction of target-cell-specific immunotoxins, either the B-chain must be omitted or its galactose-binding sites must be blocked, to prevent stray intoxication. In addition, both ricin and immunotoxins prepared with native whole ricin or ricin A-chain are rapidly cleared from the circulation by mannose-specific uptake in non-parenchymal liver cells (NPC) (Fodstad et al., 1976; Skilleter et al., 1981; Worrell et al., 1986; Bourrie et al., 1986). The consequences of this rapid clearance, i.e. short circulation half-life and concurrent hepatoxicity, pose severe limitations on the clinical usefulness of such immunotoxins. These obstacles may be circumvented to some extent by chemical or enzymic modification of the ricin oligosaccharides, which markedly decreases the association of ricin and its derived immunotoxins with NPC, both *in vivo* (Thorpe et al., 1985; Skilleter et al., 1985; Blakey et al., 1987) and *in vitro* (Skilleter and Foxwell, 1987; Blakey et al., 1988). Yet further clarification of the biodistribution and intoxication of ricin is a prerequisite for its successful application for therapeutic purposes.

An unresolved issue regarding ricin biodistribution has been which of the NPC types participate in the mannose-specific clearance of ricin. The sinusoidal endothelial liver cells (EC), which make up the characteristic fenestrated lining of the liver sinusoids, express several endocytic receptors, including mannose receptors, and play a central role in removing several hazardous compounds from the circulation by efficient endocytosis (reviewed by Smedsrød et al., 1990). On the other hand, most of the current knowledge about mannose receptors and their functions stems from extensive studies on macrophages (Stahl et al., 1984; Ezekowitz and Stahl, 1988), and researchers therefore initially tended to attribute the hepatic clearance of mannose-

Abbreviations used: AM, alveolar macrophages; EC, rat liver endothelial cells; KC, Kupffer cells; NPC, rat liver non-parenchymal cells; PC, rat liver parenchymal cells; TC, tyramine–cellobiose.

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terminated glycoconjugates to the liver macrophages, the Kupffer cells (KC) (Schlesinger et al., 1978; Furbish et al., 1981). It has later been shown that the EC are much more efficient than the KC in endocytosing different mannose-terminated compounds both *in vivo* (Hubbard et al., 1979; Niesen et al., 1984; Kindberg et al., 1990) and *in vitro* (Summerfield et al., 1982; Magnusson and Berg, 1989; Kindberg et al., 1990), and they are therefore obvious candidates for mediating the clearance of ricin as well. Early morphological studies suggested that both KC and EC were poisoned and became necrotic during the initial phase of ricin intoxication, whereas the liver parenchymal cells (PC) remained apparently intact much longer (Derenzini et al., 1976). Later, it was reported that, after intravenous injection of ricin A-chain into rats, the circulation half-life of mannose-terminated glycoproteins was markedly increased, indicating that the cells responsible for the clearance of these proteins (i.e. mainly EC) had been functionally depleted from the animals (Simmons et al., 1987). Still, Bingen et al. (1987) claimed that KC, but not EC, are damaged by ricin intoxication, and it has also been reported that KC are selectively depleted from mouse liver upon injection of ricin (Zenilman et al., 1989). As the participation of the EC in the clearance of ricin from the circulation has not been determined, it might therefore appear to be a prevailing assumption that this is predominantly carried out by the KC (Skilleter et al., 1981; Bingen et al., 1987; Zenilman et al., 1989).

We have shown previously that isolated EC endocytose ricin via both mannose receptors and surface galactosyl residues *in vitro* (Magnusson et al., 1991). In the present study we have investigated the distribution of ricin among different organs and different liver cell types after intravenous injection and uptake *in vivo* in the rat. Moreover we have studied the carbohydrate-specificity of ricin uptake by different liver cell types *in vitro*. Finally, we have studied the expression of mannose receptors in NPC and the characteristics of ricin binding to these receptors.

MATERIALS AND METHODS

Animals and reagents

Male Wistar rats weighing 200–250 g were used for all experiments. The animals were fed on standard laboratory pellets *ad lib*.

BSA, collagenase (type I) and yeast mannan were obtained from Sigma (St. Louis, MO, U.S.A.). $Na^{125}I$ was obtained from Amersham International (Amersham, Bucks., U.K.). Cell culture dishes (6.0 cm diameter) were obtained from Costar (Cambridge, MA, U.S.A.), and cell culture medium (minimal essential Eagle's medium and Dulbecco's modified Eagle's medium), foetal-calf serum and L-glutamine were obtained from Flow Laboratories (Irvine, Ayrshire, Scotland, U.K.). Ultroser G was obtained from IBF Biotechnics (Villeneuve-la-Garenne, France) and gentamicin was obtained from Gibco BRL (Uxbridge, Middx., U.K.). Immobilon membranes were obtained from Millipore (Bedford, MA, U.S.A.), and anti-rabbit IgG ABC-kit for immunoblot development was obtained from Vector Laboratories (Burlingame, CA, U.S.A.). Human fibronectin was a gift from Dr. Bård Smedsrød, Institute for Medical Biology, University of Tromsø (Tromsø, Norway), and tyramine–cellobiose (TC) was a gift from Dr. Helge Tolleshaug, Nycomed A/S (Oslo, Norway). Ricin, prepared as described by Nicolson and Blaustein (1972), and rabbit antiserum against ricin were kindly provided by Dr. Jean-Pierre Frénoy, UFR Biomédicale des Saints Pères (Paris, France). Rabbit antiserum against mannose receptors isolated from rat alveolar macrophages (Haltiwanger and Hill, 1986a) was a gift from Dr. Robert L. Hill, Duke University (Durham, NC, U.S.A.). All additional chemicals were of analytical grade.

Radiolabelling of ricin

Ricin was labelled by derivative formation with radioiodinated TC as described by Pittman et al. (1983). The labelled TC adducts are incapable of crossing cellular membranes, and thus provide a marker for the site of uptake even after the protein has been degraded. Briefly, 100 nmol of TC in 20 mM phosphate buffer, pH 7.4, was made to react with 1 mCi of ^{125}I in an Iodogen-coated tube. The reaction was stopped after 30 min by transfer to another tube containing 10 μl of 0.1 M $NaHSO_3$ and 5 μl of 0.1 M KI. Then 100 nmol of cyanuric chloride (dissolved in acetone) was added, and after incubation further for 3 min this reaction mixture was added to 1.2 mg (20 nmol) of ricin in 250 μl of 0.2 M Na_2CO_3, pH 9.5, containing 100 mM β-methyl D-galactoside in order to protect the galactosyl-binding sites from modification. After incubation at room temperature for 2 h, free ^{125}I and ^{125}I-TC were removed from the labelled ricin (^{125}I-TC-ricin) by gel filtration on a PD-10 column. The specific radioactivity was routinely in the range $(0.9–1.2) \times 10^6$ c.p.m./μg. ^{125}I-TC-ricin was bound and internalized by isolated EC with efficiency and carbohydrate-specificity identical with that for ricin labelled directly in the protein moiety by the Iodogen method (results not shown).

Intravenous injection and distribution of ricin *in vivo*

Rats were anaesthetized with pentobarbital and 10 μg (160 pmol) of ^{125}I-TC-ricin was injected in the left femoral vein, alone or together with 125 μmol of lactose, 1 mg of yeast mannan or both. After 30 min the animals were killed and the whole body was perfused with PBS through the venta porta for 2 min, to flush out the blood from the circulation. Samples of blood and liver and the entire spleen, kidneys, lungs and heart were removed and assayed for radioactivity. Different liver cell types were isolated from the remainder of the liver and established as essentially pure monolayer cultures as described below. After counting the cells in each culture under an inverted microscope equipped with a calibrated ocular insert, the cultures were solubilized with 0.1 M NaOH/0.1 % SDS and assayed for radioactivity. The total radioactivity associated with each liver cell population was calculated from the radioactivity recovered per culture divided by the number of cells in the culture and multiplied by the number of cells of each type per g wet wt. of liver and the liver wet wt. The number of the different liver cell types per g wet wt. of liver were taken to be 125×10^6 PC, 39×10^6 EC and 16×10^6 KC (Blomhoff et al., 1982; Pertoft and Smedsrød, 1986).

Preparation and culture of cells

Rat livers were perfused with collagenase as described by Berg and Blomhoff (1983) and the liver cells were dispersed in incubation buffer (Tolleshaug et al., 1977) containing 1 % BSA. PC were sedimented by centrifugation for 2 min at 16 *g* and washed twice by resuspending in the same buffer and repeating the centrifugation. Contaminating NPC were quantitatively removed by centrifugal elutriation at 1200 rev./min in a Beckman J-6M/E centrifuge equipped with a Beckman JE-5.0 elutriation rotor. NPC and cell debris eluted at a flow rate of 30 ml/min were discarded, and an essentially pure PC fraction was then eluted by increasing the flow rate to 60 ml/min. NPC were sedimented from the supernatants from the initial centrifugation and the first wash by centrifugation for 4 min at 310 *g*, resus-

pended in incubation buffer containing 1% BSA, and contaminating PC were quantitatively removed by centrifugal elutriation. NPC were collected at a rotor speed at 1500 rev./min and a flow rate of 25 ml/min, and anything eluted at a higher flow speed was discarded. After sedimenting the NPC by centrifugation for 4 min at 310 *g*, they were separated further by centrifugal elutriation at 2500 rev./min. After collection of an EC-enriched fraction at a flow rate of 22 ml/min, a KC-enriched fraction was eluted by increasing the flow rate to 60 ml/min.

Monolayer cultures of liver cells were established by suspending the enriched elutriation fractions in culture medium and plating on culture dishes as follows: PC were suspended in Dulbecco's modified Eagle's medium supplemented with 2% Ultroser G, 2 mM L-glutamine and 50 μg/ml gentamicin, and EC were suspended in minimal essential Eagle's medium supplemented with 2 mM L-glutamine and 50 μg/ml gentamicin. PC and EC were plated on culture dishes coated with fibronectin (1 μg/cm^2). After 2 h at 37 °C, non-adherent cells were removed by repeated washing with PBS. KC were suspended in minimal essential Eagle's medium supplemented with 10% foetal-calf serum and plated on culture dishes coated with glutaraldehyde-fixed BSA as described by Laakso and Smedsrød (1987). After 30 min at 37 °C, non-adherent cells were removed by repeated washing with PBS.

The average number of cells grown per cm^2 was 1.3×10^5 PC, 1.1×10^5 EC and 5.4×10^4 KC. All cultures were essentially free from contamination by other liver cell types, as judged by their morphological appearance. For further purity control, EC and KC cultures were fixed by immersion in methanol at −20 °C and incubated with the monoclonal antibody ED2 (Serotec, Oxford, U.K.), which specifically binds to rat macrophages, followed by incubation with fluorescein isothiocyanate-conjugated anti-mouse IgG antibody. By this method, KC cultures were consistently labelled, whereas no labelling was observed in EC cultures (results not shown).

Rat alveolar macrophages (AM) were obtained by pulmonary lavage with 0.9% NaCl.

Endocytosis of ricin by liver cells *in vitro*

Monolayer cultures of PC, EC and KC were incubated for 1 h with 10 nM ^{125}I-TC-ricin in incubation buffer (Tolleshaug et al., 1977) containing 1% BSA, with or without 50 mM lactose, 1 mg/ml yeast mannan or both inhibitors added. The cultures were washed three times with ice-cold incubation buffer containing 100 mM lactose and 5 mM EGTA, solubilized in 0.1 M NaOH containing 0.1% SDS and assayed for radioactivity.

Electrophoresis and blotting

AM and monolayer cultures of PC, EC and KC were solubilized in solubilizing buffer (50 mM Tris, 100 mM NaCl, 2 mM EGTA, 1 μM phenylmethanesulphonyl fluoride and 1% Triton X-100) on ice for 10 min, and insoluble material was removed by centrifugation at 8900 *g* for 10 min. Solubilized cell proteins were separated by non-reducing SDS/PAGE in 7.5% acrylamide gels as described by Laemmli (1970) and transferred on to Immobilon membranes in a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad) at 30 V for 18 h. Non-specific binding of proteins was blocked by incubating blots in 0.05% Tween 20 in the appropriate incubation buffer for 1 h before incubation with antibody or ricin and including Tween 20 at this concentration in all subsequent incubation solutions. Mannose-receptor protein was detected on the blots by incubating them with rabbit antiserum against mannose receptors from rat AM (at 1:800 dilution in PBS), followed by detection with an ABC-kit (Vector) according to the manufacturers' recommendations. Alternatively, blots were probed for ricin-binding proteins by incubating them with 10 μg/ml ricin in incubation buffer (Tolleshaug et al., 1977) containing 8 mM $CaCl_2$ and 100 mM lactose, with or without 5 mg/ml yeast mannan or 20 mM EGTA added. Bound ricin was detected on the blots by incubating them with rabbit antiserum against ricin (at 1:800 dilution in 20 mM Hepes/8 mM $CaCl_2$, pH 7.6) and detected as described for mannose-receptor immunoblots.

RESULTS

Organ distribution of intravenously injected ricin

Figure 1 shows organ distribution of ^{125}I-TC-ricin 30 min after intravenous injection. In control animals (no inhibitor present), ricin was effectively cleared from the circulation, with only 15% remaining in the blood at this time point. Over 50% of the injected ^{125}I-TC-ricin was recovered in the liver, whereas only minor amounts were recovered in spleen, kidneys and lungs. When the galactose-binding sites of ricin were blocked by co-injection of lactose, clearance and liver uptake were markedly enhanced and association with lungs was completely abolished. Co-injection of yeast mannan, which competitively inhibits binding of ricin to mannose receptors, strongly decreased the clearance and liver uptake, whereas administration of both inhibitors together had an intermediary effect.

Distribution of intravenously injected ricin among liver cell types

Figure 2 shows the distribution of ^{125}I-TC-ricin among different liver cell populations 30 min after intravenous injection. In control animals, most of the recovered ^{125}I-TC-ricin was associated with the EC, but much less was recovered in PC and KC. The carbohydrate-dependency of the EC uptake was identical with the pattern observed for uptake in whole liver, i.e. the uptake was markedly enhanced by co-injection of lactose and strongly decreased by co-injection of mannan. Uptake in PC was

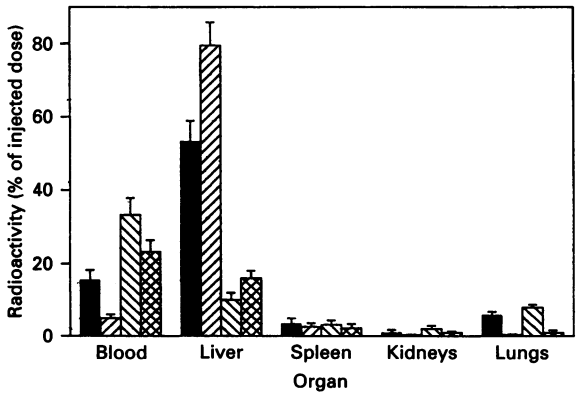

Figure 1 Distribution of ricin among rat organs 30 min after intravenous injection

^{125}I-TC-ricin (10 μg; 160 pmol) was injected intravenously into rats, alone (■) or together with 125 μmol of lactose (▨), 1 mg of mannan (▧), or both inhibitors simultaneously (⊠). After 30 min, radioactivity recovered in blood, liver, spleen, kidneys and lungs was calculated and expressed as percentage of the injected dose. The Figure shows means ± S.E.M. for three identical experiments.

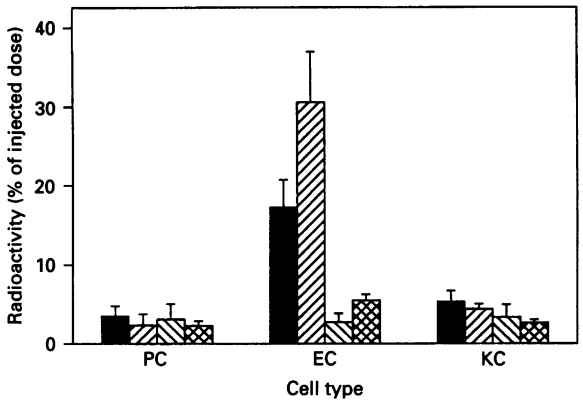

Figure 2 Distribution of ricin among rat liver cell types 30 min after intravenous injection

^{125}I-TC-ricin (10 μg; 160 pmol) was injected intravenously into rats, alone (■) or together with 125 μmol of lactose (▨), 1 mg of mannan (▧), or both inhibitors simultaneously (⊠). After 30 min the liver was perfused, and PC, EC and KC were isolated as described in the Materials and methods section. The radioactivity recovered in each cell population was calculated and expressed as percentage of the injected dose. The Figure shows means ± S.E.M. for three identical experiments.

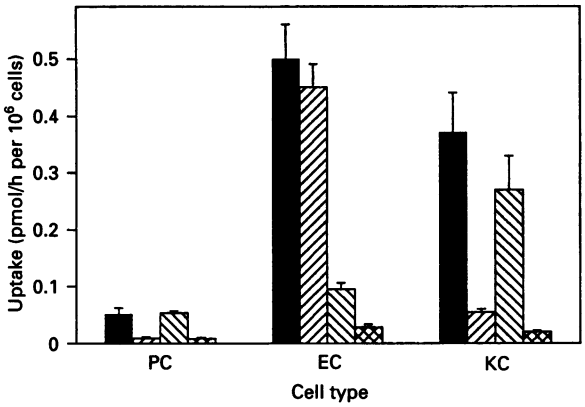

Figure 3 Endocytosis of ricin by monolayer cultures of liver cells

Essentially pure monolayer cultures of PC, EC and KC were established and incubated at 37 °C with 10 nM ^{125}I-TC-ricin, alone (■) or in the presence of 50 mM lactose (▨), 1 mg/ml mannan (▧), or both inhibitors simultaneously (⊠). Cell-associated radioactivity was measured after 1 h, as described in the Materials and methods section. The Figure shows means ± S.E.M. for three identical experiments.

partly inhibited by lactose, whereas uptake in KC was partly inhibited by both inhibitors in an additive manner.

Endocytosis of ricin by monolayer cultures of different liver cell types

Clearance of ricin by endocytosis *in vivo* is a multi-component process, involving binding to and uptake by several cell populations via either or both of the two described carbohydrate-specific mechanisms. These multiple pathways are highly interactive in the sense that perturbations in the contribution of one pathway, by co-injection of a competitive carbohydrate inhibitor, will render more ricin available for uptake by another pathway and thus influence not only the relative, but also the absolute, contribution of the latter pathway. Moreover, a minor pathway, although efficient by itself, may be grossly underestimated, or even overlooked altogether, in a multi-component system dominated by another pathway of even higher efficiency. In view of this, the carbohydrate-specificity of the uptake by each of the cell populations involved is not necessarily reflected by the carbohydrate-dependency of the biodistribution of ricin, and may only be fully elucidated in a simplified experimental system, i.e. with pure preparations of each cell population *in vitro*.

PC, EC and KC in monolayer cultures *in vitro* were all found to endocytose ^{125}I-TC-ricin, albeit with different efficiency and carbohydrate-specificity (Figure 3). In the absence of carbohydrate inhibitors, ricin was efficiently taken up by EC and only slightly less by KC, whereas relatively little was taken up by the PC (expressed per cell number). Although the uptake in EC and KC took place via both mannose- and galactose-specific mechanisms, the relative contributions of the two pathways differed strongly between the two cell types; the uptake in EC was largely mediated by mannose receptors, whereas most of the uptake in KC was galactose-specific. The slight uptake observed in PC was entirely galactose-specific.

Expression of mannose receptors in liver cells

Although both EC and KC endocytose ricin via mannose receptors, this pathway of uptake is much more efficient in EC

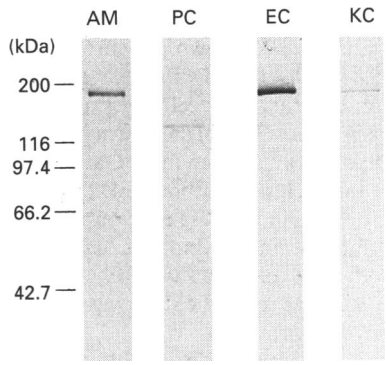

Figure 4 Expression of mannose receptors in liver cells

Solubilized protein from rat AM (75 μg) and essentially pure cultures of PC (125 μg), EC and KC (25 μg each) was subjected to non-reducing SDS/PAGE and electroblotted on to Immobilon membranes. Mannose-receptor protein was detected on the blots as described in the Materials and methods section.

than in KC, both *in vivo* and *in vitro*. This difference could be related to either different levels of expression of the mannose receptor in the two cell types or differences in the efficiency of the endocytotic machinery. As both EC and KC are known to carry out effective endocytosis, both *in vivo* and *in vitro*, it was decided to study the expression of the mannose receptor in these cell types.

Figure 4 shows a Western blot of solubilized protein from AM, PC, EC and KC. Mannose-receptor protein was detected on the blot by incubation with antiserum raised against the mannose receptor ($M_r \sim 180000$) isolated from rat AM (Haltiwanger and Hill, 1986a). At $M_r \sim 180000$, a single strong band was detected in AM and EC, whereas a much weaker band was observed in KC and no band of this M_r was observed in PC. Although no other bands were observed in AM, EC or KC, two weak bands of $M_r \sim 130000$ and 80000 were observed in PC. Incubating blots with pre-immune rabbit serum instead of antiserum to mannose receptor yielded no bands in AM, EC or KC, whereas the same weak bands were observed in PC, indicating that they were of non-specific nature (results not shown).

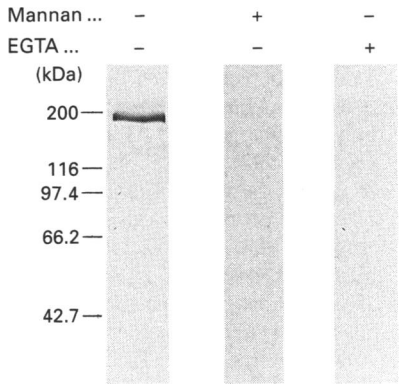

Figure 5 Characteristics of ricin binding to EC mannose receptors

Essentially pure monolayer cultures of EC were solubilized with Triton X-100, subjected to non-reducing SDS/PAGE and electroblotted on to Immobilon membranes. The blot was probed for ricin-binding proteins in the presence of 100 mM lactose and 8 mM Ca^{2+}, with 5 mg/ml mannan or 20 mM EGTA added as indicated, as described in the Materials and methods section.

Characteristics of ricin binding to mannose receptors

In addition to mannose receptors, other endocytic receptors of similar or even partially overlapping specificity have been described in NPC (Haltiwanger and Hill, 1986b; Jansen et al., 1991). In order to determine whether mannose-specific binding and uptake of ricin by EC and KC was in fact mediated by mannose receptors, Western blots of solubilized protein from EC, KC and AM were probed with ricin in the presence of 100 mM lactose, to prevent binding to any galactose-containing glycoprotein, followed by immunoblotting with anti-ricin antiserum (Figure 5). Ricin was found to bind strongly to a band of the same M_r as the mannose receptor, in EC (lane 1) as well as in KC and AM (results not shown). No other bands were observed in any of the three cell types. Binding to the EC band was completely abolished when 5 mg/ml yeast mannan (lane 2) or 20 mM EGTA (lane 3) was included in the ricin-binding step. The same binding characteristics were also observed in KC and AM (results not shown).

DISCUSSION

In the present study we show that, after intravenous injection, ricin rapidly associates with the liver in a carbohydrate-dependent manner. Among the liver cell types, ricin mainly associated with the EC in a mannose-specific manner, and to a much less extent with KC and PC. *In vitro*, endocytosis of ricin by EC was also mainly mannose-specific, whereas most of the uptake in KC was galactose-specific. This is compatible with our finding that mannose receptors were expressed at a much higher level in EC than in KC.

It has been reported previously that after intravenous injection into rats, ricin associated predominantly with the liver (Thorpe et al., 1985; Skilleter and Foxwell, 1987; Ramsden et al., 1989; Frénoy et al., 1992), but to our knowledge only one study has previously addressed the carbohydrate dependency of the total liver uptake of ricin *in vivo* (Frénoy et al., 1992). Moreover, it has been shown that *in vivo* more ricin associated with NPC than PC, and that *in vitro* uptake of ricin by NPC was mainly mannose-specific (Skilleter et al., 1981, 1985; Thorpe et al., 1985), although great care should be taken in attempts to extrapolate findings *in vitro* to the more complex situation *in vivo*. Also, previous studies of the uptake of ricin *in vivo* by rat liver have made no attempt to determine the relative contributions of the different NPC types to this process.

The data presented here on the distribution of ^{125}I-TC-ricin between rat organs in the absence of carbohydrate inhibitors agree well with previous reports (Thorpe et al., 1985; Skilleter and Foxwell, 1987; Ramsden et al., 1989; Frénoy et al., 1992). Also in good agreement with Frénoy et al. (1992), we found that most of the total liver uptake was mannose-specific. As reported previously for the uptake *in vivo* of other mannose-terminated glycoconjugates (Hubbard et al., 1979; Kindberg et al., 1990), the mannose-specific uptake of ricin took place mainly in the EC, and altogether the EC were more efficient in uptake of ^{125}I-TC-ricin *in vivo* than were both PC and KC. Enhancement of total liver uptake of ^{125}I-TC-ricin in the presence of lactose was also observed by Frénoy et al. (1992). This enhancement, which we observed both in total liver and EC, is most readily explained by inhibition by lactose of ricin binding and uptake by blood cells and other tissues that do not express mannose receptors, thus rendering more ricin available for efficient uptake via EC mannose receptors. Taken together, the carbohydrate-dependency of the EC uptake was clearly reflected in the carbohydrate-dependency of the total liver uptake, further confirming that the EC are the predominating liver cell type in ricin uptake *in vivo*.

The combined recovery of injected ^{125}I-TC-ricin in all three liver cell types did not add up to yield the amount recovered in whole liver. A number of different explanations may be proposed for this phenomenon, e.g. experimental errors in counting of cultured cells, inaccurate values for number of cells of each type per g wet wt. of liver, and uptake by other liver cell populations than those examined. However, as the procedures for isolation of PC and EC include incubation at 37 °C for up to 2 h, we suggest that this discrepancy may be explained by a toxic effect of ricin on the cells during this incubation, resulting in leakage of ^{125}I-TC-ricin from damaged cells. It is also conceivable that such an effect could be selective, i.e. if one cell type is more susceptible to poisoning and to leakage of the radioactive label during isolation than the other cell types, then the estimate of the relative contribution of the different cell types would be biased. We have shown previously (Magnusson et al., 1991) that, in terms of ricin concentration, the EC are much more sensitive to ricin toxicity than has been reported for the PC (Decastel et al., 1989). Also, we have shown that the time course of ricin toxicity, measured by effect on protein synthesis, is very rapid in the EC, allowing for a considerable effect to be observed during a 2 h incubation (Magnusson et al., 1993). In view of this and the present finding that *in vivo* the EC accumulate more ^{125}I-TC-ricin per cell than do the other liver cell types examined, it is likely that the EC are more prone to damage due to poisoning during the isolation procedure than are the other cell types examined. This was also indicated by the sub-optimal morphological appearance of EC cultures from animals injected with ^{125}I-TC-ricin alone or together with lactose as compared with cultures from animals that were either not injected or injected with ^{125}I-TC-ricin together with mannan. Cultures of PC and KC did not show any treatment-dependent morphological differences. In conclusion, the relative contribution of the EC to uptake of ^{125}I-TC-ricin *in vivo*, although major, may be underestimated.

Under control conditions *in vivo*, i.e. when no inhibitors were co-injected, more ricin was taken up in the EC than in the KC. Even when the relative numbers of cells of each population were taken into account (39×10^6 EC and 16×10^6 KC per g wet wt. of liver; Blomhoff et al., 1982; Pertoft and Smedsrød, 1986), about 30% more ricin was taken up per cell in the EC than in the KC. This is not easily reconciled with earlier claims that KC were

selectively depleted from the liver upon ricin injection, whereas EC were much less affected (Bingen et al., 1987; Zenilman et al., 1989). However, two alternative explanations may be proposed. Firstly, those earlier studies were not primarily concerned with the distinction between EC and KC and may not have applied adequate means to distinguish between the two cell types. Secondly, it is conceivable that the KC are even more sensitive to ricin *in vivo* than are the EC, due to either differences in the protein-synthesis machinery itself or functions relying on continuous protein synthesis, leading to more rapid death and disintegration of KC than of EC upon exposure to ricin *in vivo*, in spite of a less efficient total uptake.

Except for our recent report (Magnusson et al., 1991), all previous studies of ricin uptake by rat NPC *in vitro* have been carried out with total NPC preparations (Skilleter et al., 1981, 1985; Skilleter and Foxwell, 1987), and have therefore not distinguished between the different NPC types, in terms of neither uptake efficiency nor the carbohydrate-specificity of the uptake. The present data show that both the efficiency and the carbohydrate-specificity of ^{125}I-TC-ricin uptake by EC in monolayer culture are similar to results obtained with freshly isolated EC in suspension (Magnusson et al., 1991). Also, although the KC do not contribute much to the total liver uptake of ricin *in vivo*, they are nevertheless capable of efficient endocytosis of ^{125}I-TC-ricin *in vitro*. In agreement with the results *in vivo*, mannose-specific uptake in the KC was much lower than in the EC, but it was interesting that, in contrast with the EC, the galactose-specific uptake of ricin in KC was approx. 5 times the mannose-specific uptake. This is analogous to the previous observation by Simmons et al. (1986), who found that, although rat bone-marrow macrophages carry mannose receptors, internalization of ricin in these cells takes place mainly via surface galactosyl residues. Since ricin-binding galactosyl residues are likely to be evenly distributed throughout cell surfaces, the galactose-specific uptake of ricin may be used as an indicator of total membrane internalization. The galactose-specific uptake of ^{125}I-TC-ricin by KC, which is very efficient compared with both PC and EC, and by bone-marrow macrophages (Simmons et al., 1986), may thus reflect the extensive membrane internalization that takes place constitutively in KC and other phagocytic cell types. By the same measure, membrane internalization in EC is much less extensive than in KC, indicating that the efficient mannose-specific uptake in the EC is highly selective. This agrees well with our finding that, in EC, uptake of ricin via mannose receptors mainly takes place from coated pits, whereas uptake via surface galactosyl residues is largely independent of coated pits (Magnusson et al., 1991).

A possible explanation of the difference in efficiency of mannose-receptor-mediated endocytosis in EC and KC is that, whereas KC and other phagocytic cell types constitutively internalize large amounts of membrane in a non-selective manner, they have a much less efficient machinery for selective receptor-mediated endocytosis via coated pits than the EC. Although kinetic parameters of mannose-receptor internalization have not been determined in KC, an analogy may be drawn to macrophages, in which the extent of membrane internalization has also been found to be much greater than the extent of mannose-receptor-mediated endocytosis (Simmons et al., 1986). In macrophages, the efficiency of mannose receptor internalization, as measured by the endocytotic rate constant, K_e (Wiley and Cunningham, 1982; Opresko and Wiley, 1987), has been reported to be very high (Hoppe and Lee, 1983; Ward and Kaplan, 1990), i.e. of the same order of magnitude as we have observed previously in the EC (Magnusson and Berg, 1989). As this renders unlikely an explanation based on major differences in internalization efficiency, the inefficient mannose-specific uptake of ^{125}I-TC-ricin by KC, as compared with EC, prompted our study of the relative level of expression of mannose receptors in the two cell types.

Endocytic mannose receptors have been isolated from several sources, including rat (Haltiwanger and Hill, 1986a), human (Stephenson and Shepherd, 1987) and rabbit (Lennartz et al., 1987a) alveolar macrophages, as well as human placenta (Lennartz et al., 1987b). The receptors from all these sources are transmembrane glycoproteins of M_r 175000–180000, with similar binding specificity and characteristics. On the other hand, the mannose receptors of NPC have not been characterized, and there has been some uncertainty about whether they are identical with the macrophage mannose receptors. Haltiwanger and Hill (1986b) demonstrated that antiserum against the receptor from rat alveolar macrophages also reacted with both EC and KC, suggesting that the hepatic mannose receptors were identical with the macrophage receptors. However, the identity of the EC and KC protein(s) recognized by this antiserum has not previously been established. Our present data show that this antiserum reacts with protein of $M_r \sim 180000$ in all three cell types (AM, EC and KC). As equal amounts of EC and KC protein were applied to the SDS/PAGE gels, the Western blot, although semi-quantitative, clearly demonstrates that the EC express mannose receptors at a considerably higher level than the KC, expressed per cell protein. In addition, a protein of the same M_r was the only EC protein that could be shown to bind ricin in the presence of lactose, within the detection limits of the ligand-blotting protocol used. In good agreement with the binding characteristics described for mannose receptors from various sources (Ezekowitz and Stahl, 1988), this binding was mannose-specific and Ca^{2+}-dependent. Taken together, we believe that this evidence, although indirect, confirms that the mannose receptors of alveolar macrophages and liver NPC are identical. Moreover, since both EC and KC show high endocytic activity, we believe that the much more efficient mannose-receptor-mediated endocytosis in EC, as compared with KC, may largely be explained by different levels of mannose-receptor expression.

Most studies on mannose receptors have been concerned with macrophages, and it has been claimed that expression of mannose receptors is a macrophage-specific trait (Ezekowitz and Stahl, 1988). The demonstration of mannose receptors on KC, the liver macrophages, agrees well with this, whereas the EC, which express mannose receptors at significantly higher levels than the KC, are not believed to be of monocyte/macrophage origin. However, whereas the EC are both morphologically and functionally different from vascular endothelial cells, they share several characteristic functional features with macrophages and are considered to be a part of the reticulo-endothelial system (Smedsrød et al., 1990). In view of the present results, further clarification of the possible ontogenetic relationship of the EC to macrophages and other cells of the reticulo-endothelial system is warranted.

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