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Selective uptake of ricin A-chain by hepatic non-parenchymal cells in vitro

Importance of mannose oligosaccharides in the toxin

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Free ricin A-chain was actively taken up in vitro by rat liver non-parenchymal cells but not by parenchymal cells. A-chain uptake by non-parenchymal cells could be selectively inhibited by D-mannose, L-fucose or ovalbumin and was markedly decreased after partial removal of mannose residues from the oligosaccharides present in the glycoprotein by enzymic deglycosylation. Uptake of free ricin B-chain by non-parenchymal cells was greater than that by parenchymal cells but in both cases was little influenced by enzymic deglycosylation of the glycoprotein. The results are consistent with mannose receptor recognition of ricin A-chain by non-parenchymal cells and have important implications for the clinical use in vivo of antibody-ricin A-chain conjugates in cancer therapy.

Ricin *Deglycosylation* *Mannose oligosaccharide* *(Liver cell)*

1. INTRODUCTION

Ricin is a glycoprotein (M_r 62 000) from castor beans (*Ricinus communis*) which consists of two similar-sized polypeptide subunits (A- and B-chains) linked by a disulphide bond and is a potent inhibitor of protein synthesis in eukaryotic cells. The B-chain is generally regarded as necessary for the binding of ricin to cells which is followed by transfer of the A-chain to the cytosol where it inactivates the 60 S ribosomal subunits (review [1]).

Pharmacokinetic studies on ricin have shown that the glycoprotein is rapidly removed from the blood by the reticuloendothelial cells (RES), especially by the liver [2] in which severe damage occurs to the hepatic sinusoids [3] as a result of active uptake by the non-parenchymal cell population [4]. We have established previously that liver non-parenchymal cells in vitro accumulate native ricin by a dual recognition process. The toxin predominantly binds to these cells, as with other cell types, through recognition of galactose-containing glycoproteins and glycolipids on the cell surface, but it is the selective interaction of cellular mannose receptors with the mannose-containing oligosaccharides in the toxin that predominantly stimulates endocytosis [5].

Both the A- and B-chains of ricin contain carbohydrate structures that potentially could be recognised by the RES, although not all of the oligosaccharides may be exposed in the intact toxin. The A-chain comprises 64% of an A_1 component (M_r 30 000) which contains a single carbohydrate unit of composition $(Man)_{3-4}(Fuc)_1(Xyl)_1(GlcNAc)_2$ and 34% of a heavier A_2 component (M_r 32 000) which contains an addi-

Abbreviations: GlcNac, *N*-acetyl-D-glucosamine; Xyl, D-xylose; Man, D-mannose; Fuc, L-fucose, PMSF, phenylmethylsulphonyl fluoride; PBS, phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.3)

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tional second mannose-rich oligosaccharide [6]. The B-chain (M_r approx. 30000) contains two oligomannose saccharides of composition $(Man)_{4-6}(GlcNAc)_2$ [6,7]. Involvement of the B-chain carbohydrate in the selective uptake of ricin by hepatic non-parenchymal cells might be expected from our earlier studies [4,5] but this has not yet been fully established. Furthermore, it is not known if free ricin A-chain can be taken up by the RES through recognition of its own oligosaccharide side chains. Here we have examined the uptake in vitro by different liver cell types of free ricin A-chain and free ricin B-chain both before and after modification of their carbohydrate by enzymic deglycosylation. The results show that hepatic non-parenchymal cells actively accumulate free ricin A- and B-chains and demonstrate the importance of the mannose-containing oligosaccharides present in both chains to the recognition and selective uptake of the glycoproteins by these cells.

2. MATERIALS AND METHODS

Extensive details of preparation of the various ricin components used in the present study have been published elsewhere [6] but the procedures are briefly described.

2.1. *Preparation of ricin A- and B-chains*

Ricin (electrofocusing at pH 7.2) was purified from an aqueous extract of untoasted castor bean cake, essentially as described by Nicolson and Blaustein [8]. Ricin was split into its constituent chains by reduction with 2-mercaptoethanol followed by ion-exchange chromatography on DEAE-Sepharose (Pharmacia). Contaminating ricin was removed from the A-chain by two passages through asialofetuin-Sepharose and from the B-chain by ion-exchange chromatography on Cm-Sepharose (Pharmacia) as described [6].

2.2 *Deglycosylation of A- and B-chains*

A-chain was treated with α-mannosidase (Sigma) as follows. A-chain (3 ml at 0.685 mg/ml in 0.2 M sodium citrate buffer, pH 5.5) was treated with 0.75 U α-mannosidase in the presence of 1 mM PMSF for 2 days at room temperature. Deglycosylation resulted in a decrease in M_r of the A_1-chain component by 600 and the A_2-chain component by 2000 as assessed by polyacrylamide gel electrophoresis [6]. All A-chain preparations produced comparable inhibition of protein synthesis in the reticulocyte lysate assay [9]. Deglycosylation of the B-chain was carried out by treatment at room temperature of isolated B-chain (2 mg/ml) in 0.1 M citrate buffer, pH 5.5, containing 0.2 M D-galactose, 0.25% 2-mercaptoethanol and 1 mM PMSF (Sigma), either with only α-mannosidase (0.5 U/ml) for 3 days, or with endoglycosidase H (Miles Labs, 10 mU/ml) for 2 days, followed by addition of α-mannosidase (0.18 U/ml) for a further 24 h. After deglycosylation 2 mg B-chain was filtered at a flow rate of 0.5 ml/min through 1.5 ml of a Con A-Sepharose column equilibrated with PBS containing 0.1 M galactose to remove undegraded B-chain. After treatment with α-mannosidase the B-chain showed an increase in mobility when analysed by polyacrylamide gel electrophoresis equivalent to a decrease in M_r of 1500 [6]. However, approx. 50% of the material could still be bound by Con A-Sepharose indicating an incomplete removal of mannose residues. Previous studies had shown that treatment of the B-chain with endoglycosidase H alone caused a decrease in M_r of 1200 consistent with removal of only one of the oligosaccharide chains, the other probably being protected by the polypeptide structure [6]. Sequential treatment of the B-chain with endoglycosidase H and α-mannosidase resulted in a further increase in its electrophoretic mobility (equivalent to a reduction in M_r of approx. 400) associated with removal of additional mannose residues from the endoglycosidase H-resistant oligosaccharide [6], although 25% of the material still retained its ability to bind to Con A-Sepharose.

2.3. *Uptake of toxins by liver cells in vitro*

Liver cells were isolated from adult male Wistar rats (LACP) by collagenase liver perfusion as detailed previously, using Sigma type I or Boehringer collagenase to isolate non-parenchymal and parenchymal cells, respectively [5]. Uptake of toxins (0.1 μg/ml) labelled with $Na^{125}I$ (Amersham IMS30) was measured in primary monolayer cultures incubated for 20 min at 37°C in serum-free medium (GMEM, Gibco-Europe) supplemented with 1 mM glutamine (Gibco) and 50 μg/ml gentamycin (Flow). Added sugars and

ovalbumin were obtained from Sigma. Cells were harvested and their toxin content which comprised both bound and internalized components was measured as in [5].

3. RESULTS

3.1. *Uptake of ricin A-chain by liver cells in vitro*

Free ricin A-chain was actively taken up by hepatic non-parenchymal cells in vitro and the uptake specifically inhibited (75–90%) by the presence of the simple sugars D-mannose and L-fucose or the mannose-terminated glycoprotein ovalbumin, but not by D-galactose (table 1). The carbohydrate in ricin A-chain is known not to be susceptible to removal by endoglycosidases but susceptible to removal by α-mannosidase [6]. After treatment of the free A-chain with α-mannosidase a 60% fall in uptake of the toxin by non-parenchymal cells in vitro was observed which was associated also with a marked loss of inhibition by D-mannose or L-fucose (table 1). This residual degree of inhibition was similar to that observed in the presence of D-xylose (20–25%) or other irrelevant sugars (not shown) and known from previous studies to be due to a non-specific cytostatic inhibition of uptake, probably caused by exposure of the cells to relatively high concentrations of sugars [5]. The results are therefore consistent with mannose receptor-mediated uptake of ricin A-chain by hepatic non-parenchymal cells. However, ovalbumin unexpectedly caused a similar level of inhibition in the uptake of both native A-chain and the α-mannosidase-treated material, which may reflect a fortuitous steric inhibition by the cell-bound ovalbumin of an uptake mechanism not involving the cellular mannose receptors. This conclusion was supported by the observation that uptake of both the native A-chain and α-mannosidase-treated toxin was also inhibited in the presence of lactoferrin, a glycoprotein containing peripheral L-fucose (not shown). Parenchymal cells showed only a very limited non-specific uptake of ricin A-chain (0.55–0.75 ng/10^6 cells) which was essentially unaffected by enzyme modification of the toxin or the presence of simple hexose sugars.

3.2. *Uptake of ricin B-chain by liver cells in vitro*

Ricin B-chain uptake by non-parenchymal cells was approximately twice that observed for ricin A-chain, but in contrast enzyme modification of the B-chain with α-mannosidase only slightly lowered the level of uptake (table 2). Accumulation of both untreated B-chain and the α-mannosidase-treated material, however, was most strongly inhibited (65–70%) by D-mannose, L-fucose or ovalbumin; the inhibitory effect of D-galactose being lower (approx. 40%). These results show that only partial removal of mannose residues from the B-chain by α-mannosidase [6] was insufficient to alter its recognition and uptake by non-parenchymal cells. Accordingly, it might have been expected that treatment of the B-chain with endoglycosidase H and α-mannosidase would have been more effective at lowering its uptake by non-parenchymal cells. However, only a 15% decrease in B-chain uptake was observed although inhibition by D-mannose and L-fucose was significantly decreased whilst the inhibition produced by D-galactose was

Table 1

Uptake of ricin A-chain by rat liver non-parenchymal cells in vitro

A-chain	Uptake (ng/10^6 cells)	% inhibition of uptake in the presence of				
		D-Mannose (100 mM)	L-Fucose (100 mM)	D-Galactose (100 mM)	D-Xylose (100 mM)	Ovalbumin (1 mg/ml)
Free A-chain	7.6 ± 1.7	81	75	20	25	90
α-Mannosidase-treated A-chain	3.3 ± 1.2	35	29	24	20	81

Toxin uptake was measured in monolayer cultures of 2×10^6 non-parenchymal cells which were incubated for 20 min at 37°C in 3 ml serum-free culture medium containing 0.1 μg/ml ^{125}I-labelled toxin in the presence and absence of the additions indicated. Uptake values are quoted as means ± SD for 5 separate determinations. Details of the preparation and characteristics of the toxins used are given in section 2

Table 2

Uptake of ricin B-chain by rat liver parenchymal and non-parenchymal cells in vitro

Cell type	B-chain	Uptake (ng/10^6 cells)	% inhibition of uptake in the presence of				
			D-Mannose (100 mM)	L-Fucose (100 mM)	D-Galactose (100 mM)	D-Xylose (100 mM)	Ovalbumin (1 mg/ml)
Non-parenchymal cells	free B-chain	16.8 ± 4.5	67	50	41	22	64
	α-mannosidase-treated B-chain	15.1 ± 5.1	69	57	42	23	65
	endoglycosidase H/α-mannosidase-treated B-chain-	14.2 ± 4.0	39	38	55	24	32
Parenchymal cells	free B-chain	3.1 ± 0.4	28	22	74	19	13
	α-mannosidase-treated B-chain	3.3 ± 0.7	20	23	79	23	15
	endoglycosidase H/α-mannosidase-treated B-chain	3.9 ± 0.8	17	23	80	24	13

Monolayer cultures of 1.5×10^6 parenchymal and 2.0×10^6 non-parenchymal cells were used. Other details are given in table 1 and the preparation and characteristics of the B-chain are described in section 2

marginally increased (table 2). Analysis of the B-chain after the double enzyme treatment had indicated that although this gave rise to the greatest level of deglycosylation that could be obtained in undenatured B-chain [6] it was still incomplete with a proportion of the material retaining its ability to bind to Con A-Sepharose (cf. section 2.2). This suggests that elimination of mannose-dependent uptake of ricin B-chain by non-parenchymal cells requires complete removal of both oligosaccharides from the polypeptide. Uptake of ricin B-chain by parenchymal cells was 5–7-times that measured for the A-chain but similar for both native and enzyme-treated materials and only D-galactose showed any marked inhibition of B-chain uptake. Taken together these results are consistent with an interaction of the B-chain with only galactosyl-binding sites in parenchymal cells and both galactosyl-binding sites and mannose receptors in non-parenchymal cells.

4. DISCUSSION

The most important finding of this study is that free ricin A-chain can be actively taken up by hepatic non-parenchymal cells but that it is not extensively accumulated by parenchymal cells. The results are consistent with the accumulation of ricin A-chain through recognition of its mannose oligosaccharides by the mannose receptors known to be present in non-parenchymal cells [10] and which we have shown previously to be involved in the uptake of native ricin [4,5]. These findings have considerable implications for the clinical use in vivo of antibody-ricin A-chain conjugates in cancer therapy [10–12] and support the current view [12] that significant removal from the blood of these types of immunotoxins may occur as a result of recognition of the mannose oligosaccharides in the ricin A-chain component by hepatic sinusoidal or other reticuloendothelial cells, thus severely diminishing their antineoplastic potential.

REFERENCES

[1] Olsnes, S. and Sandvig, K. (1983) in: Receptors and Recognition, Series B, vol. 15 (Cuatrecases, P. and Roth, T. eds) pp. 189–236, Chapman and Hall, London.
[2] Fodstad, O., Olsnes, S. and Pihl, A. (1976) Br. J. Cancer 34, 418–425.
[3] Derenzini, M., Bonnetti, E., Marinozzi, V. and Stirpe, F. (1976) Virchows Arch. B 20, 15–28.
[4] Skilleter, D.N., Paine, A.J. and Stirpe, F. (1981) Biochim. Biophys. Acta 677, 495–500.

[5] Skilleter, D.N., Price, R.J. and Thorpe, P.E. (1985) Biochim. Biophys. Acta , in press.
[6] Foxwell, B.M.J., Donovan, T.A., Thorpe, P.E. and Wilson, G. (1985) Biochim. Biophys. Acta 840, 193-203.
[7] Nanno, S., Ishiguro, M., Funatsu, G. and Funatsu, M. (1971) Jap. J. Med. Sci. Biol. 24, 39-42.
[8] Nicolson, G.L. and Blaustein, J. (1972) Biochim. Biophys. Acta 266, 543-547.
[9] Saragiacomo, M., Barbieri, L., Stirpe, F. and Tomasi, M. (1983) FEBS Lett. 157, 150-159.
[10] Neufeld, E.F. and Ashwell, G. (1980) in: The Biochemistry of Glycoproteins and Proteoglycans (Lennarz, W.J. ed.) ch. 6, pp. 241-266, Plenum, New York.
[11] Olsnes, S. (1981) Nature 290, 84.
[12] Vitetta, E.S. and Uhr, J.W. (1984) Transplantation 37, 535-538.