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CONCANAVALIN A MEDIATED UPTAKE OF ENZYMES BY FIBROBLASTS

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SUMMARY: Concanavalin A (Con A) enhanced the binding and uptake of glycoproteins by human fibroblasts at doses which did not affect the uptake of two non-glycosylated proteins. When Con A was preincubated with glycoproteins and the mixture later added to cultured cells, Con A-mediated uptake was time-, temperature-, and lectin-concentration dependent. α-Methylmannoside (αMM), at concentrations which have no effect on the phosphomannosyl-recognition system for uptake of acid hydrolases, inhibited Con A-mediated uptake. These studies illustrate that the analysis of adsorptive pinocytosis of glycoproteins isolated on Con A-Sepharose may be complicated by Con A-mediated uptake of glycoproteins otherwise not taken up.

INTRODUCTION: Hickman and Neufeld (1) suggested that many lysosomal hydrolases have a "common recognition marker", essential for their uptake by fibroblasts. Studies with human platelet β-glucuronidase (2) suggested that adsorptive pinocytosis of this enzyme by fibroblasts involves recognition of 6-phosphomannose residues on forms of the enzyme susceptible to pinocytosis - called "high uptake forms" (3). Extension of these findings to other hydrolases (4,5,6) led us to postulate that 6-phosphomannose was part of the common recognition marker for uptake (4). Other forms of acid hydrolases appear to lack this marker and are taken up at very low rates by fibroblasts (1,3,4,7). In fact, in β-glucuronidase extracted from most tissues, most of the catalytic activity resides in "low uptake forms" which are taken up very poorly by fibroblasts (3).

Fibroblasts from patients with I-cell disease secrete many acid hydrolases which are recognition defective (1). This was the observation that led Hickman and

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Neufeld to suggest that many acid hydrolases share a common recognition marker for uptake by fibroblasts. In the course of experiments carried out with I-cell secretion hexosaminidase in which the enzyme was isolated by adsorption to, and elution from Concanavalin A-Sepharose, we discovered an activity which appeared to convert I-cell hexosaminidase into a "high-uptake" form for fibroblasts. This activity could also convert human placental β-glucuronidase into "high-uptake" forms. The initial and incorrect interpretation was that an enzymatic activity was involved in the conversion phenomenon. Subsequent experiments led us to identify the agent that stimulates the uptake of the "low-uptake" enzymes as Con A which came off the Con A-Sepharose column with the eluted enzyme (8). In this report, we present experiments which demonstrate Con A mediated uptake of β-glucuronidase, I-cell hexosaminidase, mannose-albumin glycoconjugate (Man-BSA) and ribonuclease B (RNase B).

MATERIALS AND METHODS: Human placenta β-glucuronidase was prepared as previously described (4). N-acetyl-β-D-hexosaminidase (designated below as hexosaminidase) from I-cell fibroblast secretions was collected as described by Kaplan et al. (4), concentrated by ultrafiltration and applied to a column of concanavalin A-Sepharose (Pharmacia). After removal of unadsorbed protein, hexosaminidase activity was eluted with 0.75 M α-methyl-D-mannoside in 0.15 M NaCl, 10 mM Na_2HPO_4, 20 mM Tris-HCl buffer, pH 7.5. Hexosaminidase-free human serum albumin (The American National Red Cross) was added to the fibroblast secretion medium (final concentration 1 mg/ml) prior to ultrafiltration. Ribonuclease B (Sigma type XII-B) was repurified on concanavalin A-Sepharose to remove any ribonuclease A (RNase A) which was shown to be present (9). To eliminate possible Con A contamination both RNase A and RNase B were subjected to ultrafiltration on XM-50 membrane filters (Amicon), concentrated on UM-2 membrane filters (Amicon), chromatographed on Sephadex G-75 (1.5x58 cm) followed by ultrafiltration and exhaustive dialysis against 20 mM Na_2HPO_4, pH 7.6. Mannose-BSA (gift of Dr. Y. C. Lee) (10), bovine serum albumin,RNase A and RNase B were iodinated using the chloromine T method (11). Specific activities were 6 ± .3 μCi/μg for RNase A and B and 36±6 μCi/μg for BSA and Man-BSA. β-Glucuronidase and β-hexosaminidase-deficient human fibroblasts were grown as described previously (2).

Pinocytosis measurements: The indicated proteins were incubated with Con A (Sigma, grade IV) 20 min at room temperature in the presence of 250 μg human serum albumin, 0.15 M NaCl, 10 mM Na_2HPO_4, 10 mM Tris-HCl, pH 7.6, in a final volume of 0.2 ml. One ml of Eagle's Minimal Essential Medium containing 0.2% human serum albumin was then added and 1 ml of this mixture was layered onto cultured fibroblasts in 35 mm petri dishes. The cells were approximately 75% confluent. After 1 hr incubation at 37°C, dishes were chilled and washed six times with cold phosphate-buffered saline. Cell associated activity was measured as described by Kaplan et al. (2). β-Glucuronidase and hexosaminidase were assayed as described by Glaser et al. (12) and protein according to Lowry et al. (13). Radioactivity was measured in a Packard gamma counter.

The rate of binding and/or uptake is expressed as the percent of added radioactivity or enzymatic activity which was cell associated per unit time (% uptake/mg protein/hr).

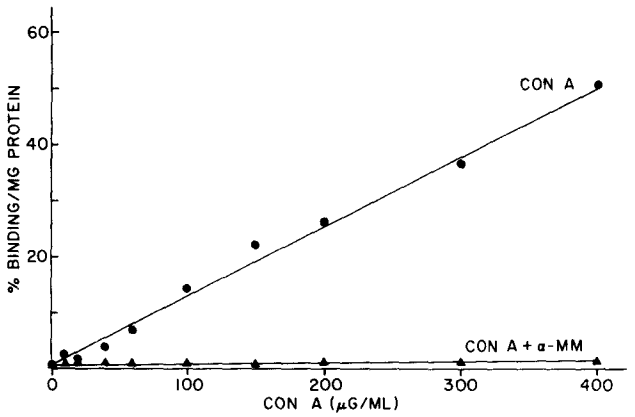

Figure 1. Cells were washed with cold Tris-phosphate buffered saline (TPBS) and then incubated with 0.5 ml of TPBS containing different concentrations of Con A with (▲) or without (●) 50 mM αMM. After 15 min at 4°C dishes were washed and incubated with 1700 U of β-glucuronidase in 0.5 ml of TPBS. At the end of the second incubation period (15 min at 4°C), plates were washed and cell associated activity was measured.

RESULTS AND DISCUSSION:

Binding of placental β-glucuronidase to Con A precoated cells - Human placental β-glucuronidase which is predominantly a "low-uptake" form for fibroblasts does not bind detectably to the cell surface of human fibroblasts. Figure 1 shows that prior treatment of human fibroblasts with Con A at 4°C and subsequent removal of unbound lectin by extensive washing, leads to dramatic binding of enzyme added to the fibroblasts in a second incubation at 4°C. The amount of enzyme bound is proportional to the Con A concentration used in the prior incubation. Essentially no enzyme binding occurred when 50 mM αMM had been present during the exposure of fibroblasts to Con A. These data suggest that the Con A serves as a bridge between cell surface glycoproteins and the "low-uptake" β-glucuronidase.

Con A mediated uptake of "low-uptake" β-glucuronidase - It has been previously established that Con A can bind to the surface membrane (14,15) and be internalized (16) by fibroblasts in culture. Human placental β-glucuronidase is itself, very poorly taken up by human fibroblasts (0.2% U/mg/h). However, mixing the enzyme with free Con A in a prior incubation and subsequent exposure of cells to

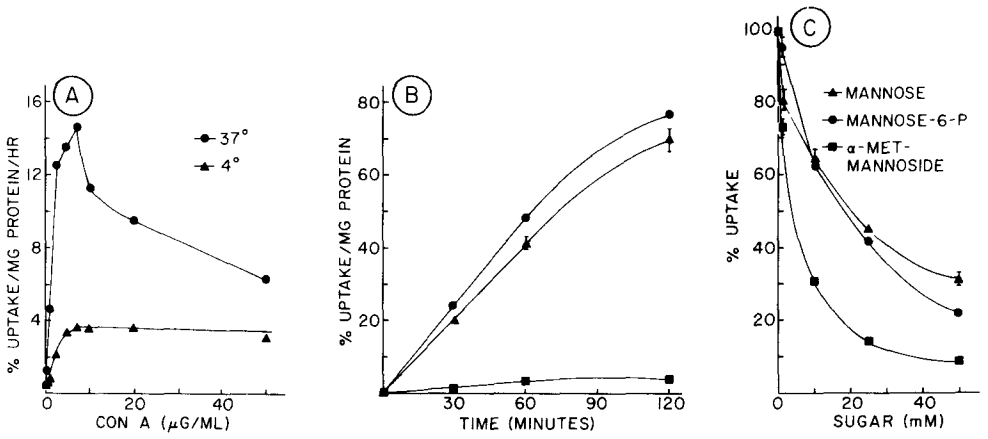

Figure 2. A. Con A mediated binding (4°C) and uptake (37°C) of β-glucuronidase. Cells in 35 mm dishes were incubated for 1 hr at 4°C (▲) and 37°C (●) with 1 ml of TPBS containing β-glucuronidase (1700 U), Con A at the concentrations indicated and human serum albumin (25 μg). The β-glucuronidase and Con A had been preincubated in 0.2 ml of TPBS for 20 min at room temperature before dilution and adding to cells.
B. Time dependence of Con A mediated enzyme uptake. Con A (5 μg), preincubated with 1600 U of β-glucuronidase was added to cells in the presence of (■) or in the absence of (● , ▲) 50 mM αMM. At the end of the incubation period cells were washed with buffered saline with (▲ , ■) or without (●) 50 mM αMM.
C. Inhibition of the Con A mediated uptake by sugars. Con A (5 μg) was preincubated with 1600 U of β-glucuronidase and then exposed to cells in the presence of mannose (▲), mannose-6-phosphate (●) or αMM (■). Cell associated β-glucuronidase was measured after a 1 hour incubation at 37°C.

the enzyme-lectin mixture produced an increase in cell-associated enzyme. Figure 2-A shows the effect of increasing concentrations of Con A on the amount of cell-associated enzyme when the cells were exposed to the mixtures at 4°C and 37°C. At 4°C, where cell-associated enzyme presumably represents enzyme bound but not internalized, maximum stimulation of binding occurred at 5-8 μg/ml Con A. Washing the dishes with PBS containing 50 mM αMM after the 1 hour incubation period at 4°C, released 29% of the cell-associated enzyme bound with 5 μg/ml of Con A. At 37°C the lectin produced a much larger increase in cell-associated enzyme. Again, the maximum stimulation occurred between 5-8 μg/ml; but higher levels led to reduced uptake. In this experiment, enzyme which became cell-associated during the 1 hour incubation at 37°C presumably reflected the sum of enzyme bound to the cell surface as a consequence of the interaction with the lectin, and enzyme internalized after

binding. In agreement with the expectation from this interpretation, only 6% of the enzyme taken up after 1 hour in the presence of 5 μg/ml Con A was releasable by washing with 50 mM αMM.

Time dependence of Con A mediated β-glucuronidase uptake - Figure 2-B shows the time dependence of Con A mediated uptake of placental β-glucuronidase at 37°C. The enzyme taken up, which includes the sum of bound and internalized enzyme, increased linearly under these conditions for at least 60 min. Addition of αMM (50 mM), at the time the presumptive enzyme - Con A complex was added to plates, largely prevented the time dependent uptake. In contrast, addition of the sugar after the incubation period released only a small fraction (6-17%) of the fibroblast associated enzyme. This observation suggested that most of the cell associated enzyme had been internalized during the incubation at 37°C.

Inhibition of the Con A mediated uptake by simple sugars - Figure 2-C compares the inhibition of placental β-glucuronidase uptake by sugars present in the medium to which the Con A/β-glucuronidase mixture was added. αMM was clearly the most potent inhibitor, about 5 fold more potent than the other sugars based on the concentration producing half of maximum inhibition. These results contrast sharply with the results of these inhibitors on the adsorptive pinocytosis of acid hydrolases by the phosphomannosyl recognition system in fibroblasts. There, mannose-6-P is about 1,000 times more potent than mannose or αMM as an inhibitor of enzyme pinocytosis (2).

Effect of Con A on the uptake of other proteins - Edelson and Cohn (17) reported that Con A produced a four fold enhancement of uptake of horseradish peroxidase (HRP) by mouse peritoneal macrophages. They did not attribute this stimulation to the interaction between Con A and the mannose-rich glycoprotein (HRP), since they observed similar stimulation of $[^{125}I]$BSA uptake.

We have examined the effects of Con A on two proteins which contain no carbohydrate, and also on several glycoproteins. In Fig. 3 we compare the stimulation of uptake by Con A for $[^{125}I]$-mannose-BSA, and $[^{125}I]$RNase B. RNase B has a single oligosaccharide chain and is quantitatively retained on Con A-Sepharose

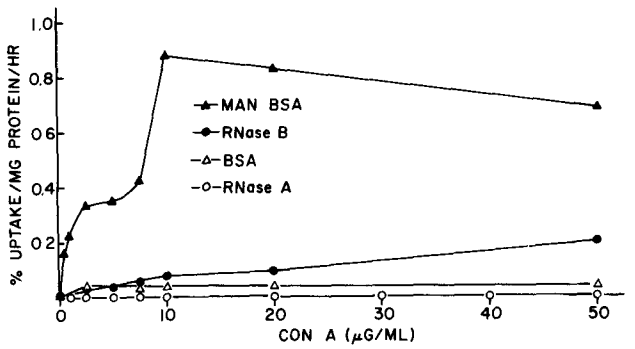

Figure 3. Effect of Con A on uptake of glycosylated and non-glycosylated proteins. Experiments were performed as described in Fig. 2, Con A was preincubated in the presence of 5 pmoles of [^{125}I] BSA (△), [^{125}I] Man-BSA (▲), [^{125}I] RNase A (○) or [^{125}I] RNase B (●), before the mixture was added to the dishes. Cell associated radioactivity measured after a 1 hour incubation at 37°C.

columns. Only 16% of the [^{125}I] Man-BSA containing an average of 20 mannose substitutions was retained by Con A-Sepharose and specifically eluted. Stimulation of uptake by Con A is seen both with [^{125}I] RNase B and [^{125}I] Man-BSA, but not with the corresponding noncarbohydrate containing proteins (Fig. 3). Note that the stimulation seen with these glycoproteins is very small compared to that for β-glucuronidase (Fig. 2) or I-cell secretion hexosaminidase (Fig. 4). Figure 4 (A-C) presents experiments showing the effect of Con A on the uptake of three different glycoproteins, RNase B, human placental β-glucuronidase, and hexosaminidase from I-cell secretions. The single oligosaccharide chain of RNase B has been well characterized (18). Human placental β-glucuronidase is a tetramer which contains 6.5% carbohydrates, and is mannose-rich (19). However, the number of oligosaccharide chains per monomer is not known. Little or nothing is known of the carbohydrate chain composition of I-cell secretion hexosaminidase. However, it is quantitatively adsorbed from culture medium by Con A-Sepharose (A. Gonzalez-Noriega and W. S. Sly, unpublished observations). The stimulation, by increasing concentrations of Con A, of uptake of these three different glycoproteins, studied either individually or in the presence of a second glycoprotein, is shown in Fig. 4. The pattern of stimulation appeared distinctive for each of the three glycoproteins studied. Addition of a second glycoprotein influenced the level of stimulation seen

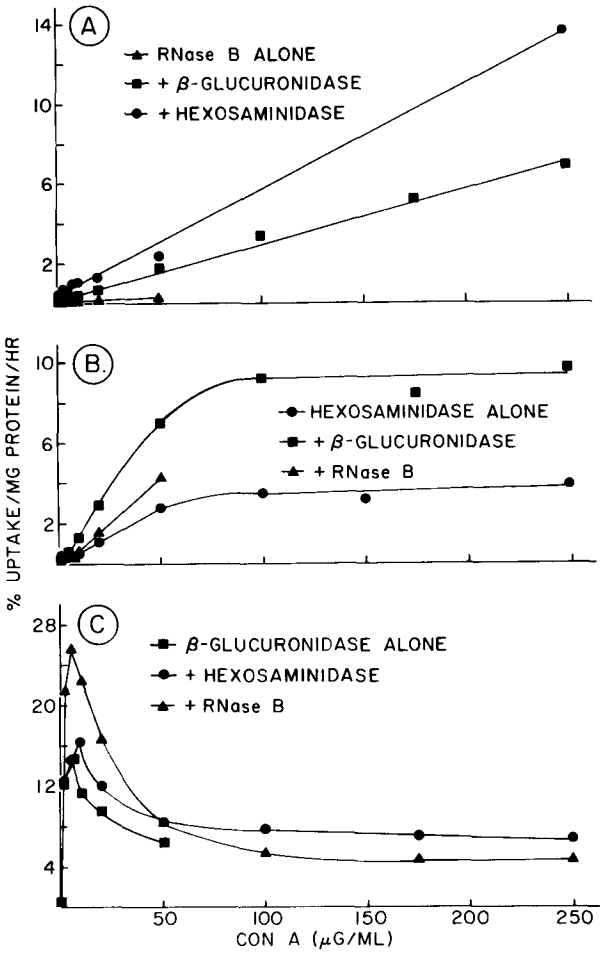

Figure 4. Percent of cell associated [^{125}I] RNase B (A), hexosaminidase (B) and β-glucuronidase (C) when the indicated concentration of Con A was preincubated with each glycoprotein singly or with one of the other two glycoproteins. Amount of each added when present: [^{125}I] RNase B (5 pmoles), hexosaminidase (1300 U) β-glucuronidase (1600 U). Cell associated enzyme activity or radioactivity were measured after a 1 hour incubation at 37°C.

(in all 3 cases enhancing uptake), but did not appear to alter the pattern of uptake of the first ligand. We have no explanation for this effect.

Con A is known to be a tetravalent lectin (20). As such, it might be expected to serve as a cross-linking agent between cell surface glycoproteins and some glyco proteins in the medium. Such reactions could be quite complicated since either the cell surface glycoprotein or the protein in the medium can be monovalent or multi-

valent itself. Presumably such differences in the enzymes explains the different patterns of stimulation by Con A.

Beeck et al. (21) reported an abstract on the effects of Con A and succinyl Con A on endocytosis of lysosomal enzymes. Their studies differed from those presented here. The enzymes used were subject to adsorptive endocytosis in the absence of lectin (i.e. were "high uptake" forms) and they described inhibition of enzyme pinocytosis by the lectins. These effects were attributed to interactions of the lectin with both the recognition marker on the enzymes and the fibroblast receptor. We intentionally restricted our studies to the effects of Con A on pinocytosis of glycoproteins which are not subject to adsorptive endocytosis. Our studies demonstrated dramatic stimulation of pinocytosis of these glycoproteins by Con A under conditions where uptake of non-glycoproteins was not affected. We interpret these observations to indicate that the lectin can serve as a bridge between a lectin binding site on the cell surface and a lectin binding site on the glycoprotein and lead to uptake of the glycoprotein by "piggyback endocytosis."

The finding that Con A can mediate uptake of a glycoprotein otherwise not susceptible to adsorptive pinocytosis has important practical implications. Many glycoproteins are isolated by affinity chromatography using Con A-Sepharose. Since free Con A is known to come off such columns (8), studies of enzyme uptake using enzymes isolated on Con A-Sepharose columns must be interpreted very cautiously. One must provide evidence that no free Con A is present in the enzyme preparation, or that the uptake is not inhibited by αMM. A practical way to distinguish Con A-mediated uptake by fibroblasts is to include 25 mM αMM in the incubation. That concentration is too low to inhibit the uptake by the 6-phosphomannosyl recognition system (2) but effectively blocks the Con A mediated uptake which we have described here.

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