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CONCANAVALIN A PROMOTES THE UPTAKE OF LYSOSOMAL HYDROLASES BY HUMAN FIBROBLASTS

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Summary

Human placental hexosaminidase B and β-galactosidase are taken up very poorly by human fibroblasts in culture. However, if fibroblasts manifesting genetically determined deficiencies of these lysosomal hydrolases are first treated with concanavalin A, then enzyme uptake is markedly increased. Enzyme activity which becomes associated with concanavalin A-treated fibroblasts maintained at 4°C can be greatly removed by treatment with haptene sugar, while enzyme activity which becomes associated with cells maintained at 37°C is refractory to haptene treatment. These results are interpreted as an initial binding of enzyme to concanavalin A molecules located at the cell surface, followed by an active cellular process leading to internalization of the lectin-enzyme complexes.

A number of attempts have been made to correct enzymatic deficiencies in genetically defective human fibroblasts by administration of exogenous enzymes [1—8]. In some cases the administered enzyme was readily taken up by the cells and served to modify cellular metabolism. However, in a number of instances [9—11], very little enzyme uptake was observed. It has recently been postulated that lysosomal hydrolases, which are mainly glycoproteins , may exist as high or low uptake forms, depending on the nature of the oligosaccharide moieties associated with these molecules [12—15]. Presumably an appropriate carbohydrate configuration must be present on the enzyme in order to promote interaction with cell surface receptors and subsequent endocytosis. In our present studies we have sought to devise a simple technique for enhancing the uptake of a variety of lysosomal hydrolases by capitalizing on the glycoprotein nature of these enzymes, and on the tendency of cells to redistribute and sometimes internalize portions of the cell surface which have been cross-linked by multivalent ligands, such as lectins [16—21]. We have treated cells with concanavalin A and subsequently exposed the lectin-treated

TABLE I

CONCANAVALIN A-MEDIATED UPTAKE OF LYSOSOMAL ENZYMES BY HUMAN FIBROBLASTS

Confluent monolayers of Sandhoff or GMI fibroblasts ($2 \cdot 10^5$ cells), in Linbro wells were incubated with 500 μg/ml concanavalin A in Dulbecco's phosphate-buffered saline for 2 h at 4°C. Excess lectin was then rinsed away and the cells were incubated with either hexosaminidase B or β-galactosidase (35 nmol/h per well) in either phosphate-buffered saline (for β-galactosidase) or Eagle's minimal essential medium (for hexosaminidase) plus 1% (hexosaminidase and β-galactosidase-free) bovine albumin for 2 h at 4°C. The monolayers were further incubated at either 4 or 37°C for an additional 16 h. At this time, some of the samples were post-treated with haptene (500 mM α-methyl mannose) in phosphate-buffered saline for 2 h at 4°C. The cells were then rinsed 3 times in phosphate-buffered saline, dislodged by scraping, lyophilized and assayed for enzyme activity as described in the text. Preliminary experiments on the removal of cell-bound ^{125}I concanavalin A by mannose or α-methyl mannoside suggested that high concentrations (greater than 200 mM) of these haptenes were required for maximal (approx. 80%) removal.

Sample	Concanavalin A	Enzyme	Haptene post treatment	Temperature (°C)	Cell-associated enzyme activity (mean ± S.E.; $n = 3$)
Hexosaminidase					
A 1	+	+	−	4	21.2 ± 2.2
2	+	+	+		1.0 ± 0.5
B 1	+	+	−	37	23.0 ± 2.1
2	+	+	+		15.3 ± 4.3
C 1	+	−		4	1.0
2	−	+			1.2 ± 0.5
β-Galactosidase					
A 1	+	+	−	4	7.3 ± 0.8
2	+	+	+		3.4 ± 0.4
B 1	+	+	−	37	7.1 ± 0.7
2	+	+	+		6.4 ± 0.8
C 1	+	−			0.4
2	−	+		37	0.9
3	−	−			0.3

cells to partially purified lysosomal enzymes which are known to bind to concanavalin A (Callahan, J., unpublished observations), namely human placental hexosaminidase B and β-galactosidase. The surface-bound concanavalin A served as an artificial cellular "receptor" for the enzyme which was then efficiently adsorbed and apparently internalized.

Human placental β-galactosidase was purified via a modification (Callahan and Gerrie, in preparation) of the method of Norden et al. [22]. The enzyme had a specific activity of 6.5 μmol/h per mg protein (410-fold purified). Human placental hexosaminidase B was prepared as described by Lowden et al. [23]. Several batches of enzyme with activities of about $2.5 \cdot 10^3$ μmol/h per mg protein were used in these studies. Assays of enzyme activities in solution and in lyophilized cell pellets were performed using 4-methyl-umbelliferyl-β-D-*N*-acetyl glucosamine (for hexosaminidase) and 4-methyl-umbelliferyl-β-D-galacto pyranoside (for β-galactosidase) as substrates and determining the quantity of methylumbelliferone produced by fluorimetry. The aberrant cell strains used in the study were HSC strain 116 (generalized gangliosidosis) and HSC strain 322 (Sandhoff's disease) and were authenticated both by clinical diagnosis and by assay of the lysosomal hydrolases. In strain 116 the level of β-galactosidase activity was 1% of that in normal fibroblasts, while in strain 322 the level of hexosaminidase activity was 0.9% of that in normal fibroblasts. Enzyme uptake experiments were performed by treating fibroblast monolayers with concanavalin A at 4°C, washing away the excess lectin and then incubating with enzyme at either 4 or 37°C (details given in figure legends).

The basic aspects of the concanavalin A-mediated enzyme uptake phenom enon are illustrated in Table I. In the case of both β-galactosidase with GMI gangliosidosis fibroblasts, and hexosaminidase B with Sandhoff's disease fibroblasts, pretreatment of the cells with concanavalin A markedly increases the amount of exogenous enzyme activity which becomes cell associated. Little difference is manifest between the total amount of enzyme activity which becomes cell associated at 4°C as opposed to 37°C. However, there is a marked difference in the accessibility of the cell-associated enzyme at the two temperatures. At 4°C, most of the enzyme, especially in the case of hexosaminidase B, can be removed by treatment with haptene sugar, while at 37°C, most of the cell-associated enzyme activity cannot be removed by haptene. This seems similar to events seen in the binding of lectins, antibodies and other multivalent ligands to cells, where the bound material remains at the cell surface at low temperature, but is internalized by an endocytotic process at physiological temperature [19]. Assuming that the difference in the amount of cell-associated enzyme removable by a haptene post-treatment in cells incubated at 4°C, as compared to that removable by haptene in cells incubated at 37°C, represents enzyme which has become internalized via active cellular processes, then 65% of cell-associated hexosaminidase and 42% of the cell-associated β-galactosidase became internalized during incubation at 37°C.

The amount of enzyme which becomes cell associated is markedly dependent upon the concentration of concanavalin A in the medium (Fig. 1). At 100—200 μg/ml of lectin, maximal uptake of enzyme is observed; this corresponds with the lectin levels at which cell surface binding sites become saturated as measured with ^{125}I concanavalin A (Juliano et al., unpublished). At greater than saturating levels of lectin (250 μg/ml), hexosaminidase B up-

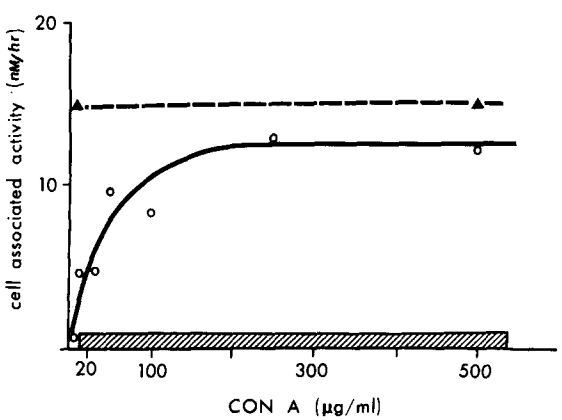
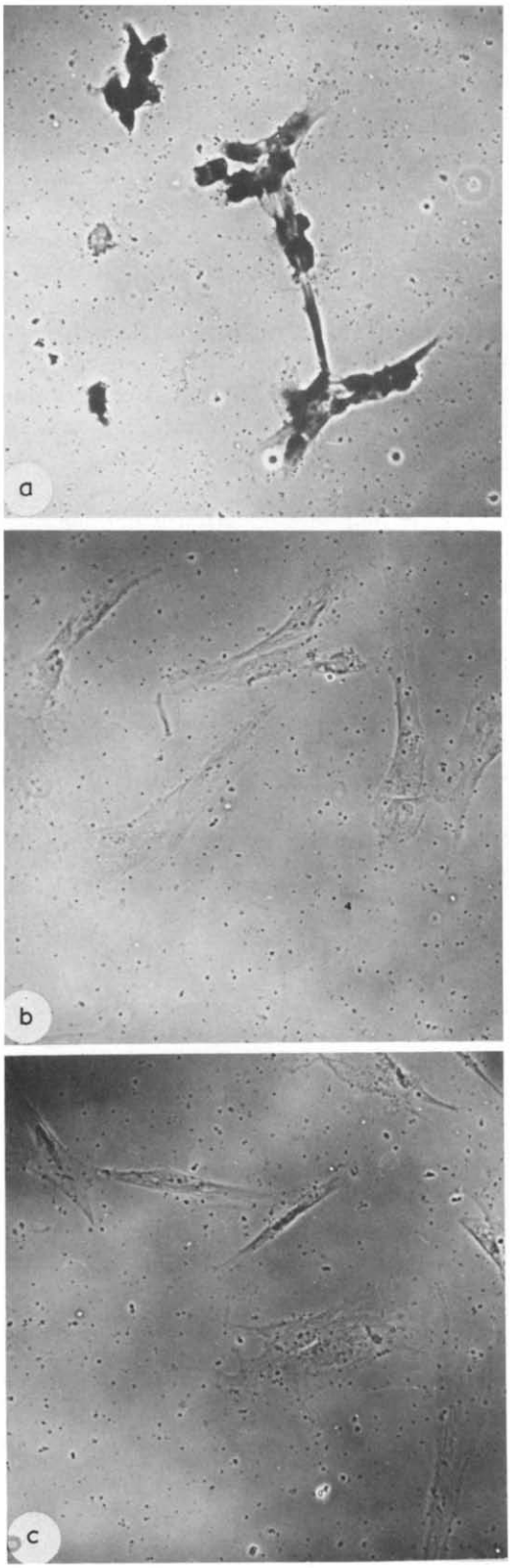


Fig. 1. Effect of concanavalin A (CON A) concentration on the uptake of hexosaminidase B. Monolayers of Sandhoff fibroblasts in Linbro wells ($2 \cdot 10^5$ cells) were treated with various concentrations of concanavalin A in phosphate-buffered saline at 4°C for 2 h. Subsequently, the monolayers were rinsed and hexosaminidase B (15 nmol/h per well), in Eagles' minimal essential medium plus 1% bovine albumin, was added to each well. The incubation was continued for a further 2 h at 4°C and thereafter the monolayers were rinsed 3 times in phosphate-buffered saline, the cells were removed by scraping, and were lyophilized and assayed for enzyme activity as described in ref. x. The data represent the means of three determinations. o—o, cell-associated hexosaminidase in the presence of concanavalin A; ▲—▲, total amount of hexosaminidase per sample. The shaded area represents the amount of enzyme activity which becomes cell associated in the presence of haptene, and in all cases was less than 10% of the added enzyme. Abscissa, μg/ml concanavalin A; ordinate, cell-associated enzyme activity in nmol/h.


a
b
c

take increases with the amount of added enzyme up to a level of 100 nmol/h per well for the preparation employed here, and thereafter the uptake apparently saturates (Juliano et al., unpublished). This apparent saturation of enzyme uptake may be due to full occupancy of available binding sites on concanavalin A molecules located at the cell surface. The maximal amount of enzyme which can become cell associated in the presence of concanavalin A is likely to vary with the purity of the enzyme preparation and with the presence of competing glycoprotein molecules. It should be noted (Fig. 1) that concanavalin A induced enzyme binding is extremely efficient with more than 85% of the total available enzyme activity becoming cell associated during a 2-h incubation at 4°C. By appropriate choice of lectin and enzyme concentrations, one can obtain levels of exogenous enzyme activity in deficient fibroblasts almost as high as the native activity found in their normal counterparts.

The enzyme activity which becomes cell associated in the presence of lectins at 37°C can readily be visualized within the cell by histochemical techniques [24]. In Fig. 2, we have utilized a chromogenic derivative of hexosamine to visualize hexosaminidase activity taken up by Sandhoff fibroblasts. Cells treated with concanavalin A and hexosaminidase B and incubated at 37°C stain intensely in this system, while cells treated with lectin plus enzyme and maintained at 4°C, or cells treated with enzyme alone at 37°C, fail entirely to stain. In the first case, the cells have had an opportunity to internalize the concanavalin A-enzyme complexes bound to their surfaces; thus the histochemical reaction product is formed within the cells and the cells are stained. In the second case, although enzyme is present on the cells (see Table I), it is presumably located at the cell surface, thus the histochemical reaction product escapes, and the cells are unstained. In the third case, insufficient enzyme is bound to the cell and little reaction product is formed.

Although it is clear that lectins can impair the long term growth properties of mammalian cells [25], treatment with concanavalin A, under the circumstances prevailing in this study, does not markedly affect the viability of human fibroblasts in the short term. Thus, in one series of experiments

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Fig. 2. Cytochemical staining of hexosaminidase B taken up by concanavalin A-treated Sandhoff fibroblasts. Subconfluent monolayers of Sandhoff disease fibroblasts (HSC strain 322) grown in tissue culture medium alpha plus 15% fetal calf serum were established in Lab Tek slide wells. The cells were rinsed three times in Dulbecco's phosphate-buffered saline and then treated with concanavalin A plus hexosaminidase B, or hexosaminidase B alone. After appropriate incubations (see below) the cells were rinsed three times in phosphate-buffered saline, fixed in 10% formaldehyde in saline and stained for hexosaminidase activity as described in ref. 24. The cells were photographed using a Leitz phase contrast photomicroscope. Hexosaminidase activity within the cells gave rise to dark blue staining of the entire cell body (seen as dark grey in the photographs); the small dark spots covering both cells and substratum represent non-specific precipitate formation which accompanies the staining reaction. (a) Concanavalin A plus Hexosaminidase B (37°C). Cells were treated with concanavalin A (200 μg/ml) in medium alpha plus 0.5% bovine albumin for 2h at 4°C. The cells were washed free of lectin and then treated with hexosaminidase B (500 nmol/h) in medium alpha plus 0.5% bovine albumin for 2 h at 4°C followed by transfer to 37°C for 12 h. (b) Hexosaminidase B (37°C). Cells were treated with hexosaminidase B (500 nmol/h) at 4°C for 2 h followed by transfer to 37°C for 12 h. (c) Concanavalin A + hexosaminidase B (4°C). Cells were treated with concanavalin A and hexosaminidase B as in (a) but were maintained at 4°C for 12 h. An intense staining reaction is evident in a but not in b or c. It should be noted (Table I) that the total amount of cell-associated hexosaminidase activity is probably the same in a and c, but only in a have the cells been given the opportunity of internalizing the enzyme. Concanavalin A-treated cells maintained at 37°C became "sticky" and tended to cluster together. This effect has also been noted by Mannino and Burger [28].

where the viability of fibroblasts was measured by dye exclusion, it was found that cells coming immediately from culture were 100% viable, while cells incubated under the conditions used by us to study enzyme uptake were 80% viable, and cells treated with 250 μg/ml concanavalin A and then incubated were 72% viable. Thus, while prolonged incubation in the absence of serum does cause some impairment of cell health, the presence of concanavalin A causes only a small additional impairment in short term viability. In this study, we are primarily interested in the fate of the cells and enzyme during the short term (1—2 days).

In summary, concanavalin A treatment of fibroblasts can dramatically promote the binding of at least two lysosomal hydrolases, and after incubation at physiological temperature, a large portion of the bound enzyme apparently becomes internalized. We interpret these observations as the formation of enzyme-lectin complexes at the cell surface followed by a gradual internalization via adsorbtive endocytosis [26]. Alternative explanations of the results shown in Table I include (1) a general increase in bulk endocytosis mediated by concanavalin A and (2) the "unmasking" of latent enzyme receptor sites by concanavalin A treatment. The first alternative is ruled out by the magnitude of the effect (up to 85% of the available enzyme is bound) which cannot be accounted for in terms of bulk endocytosis of the medium. The second alternative cannot be ruled out at this point; however, it seems redundant to postulate the existence of latent receptors when concanavalin A itself clearly has the ability to bind lysosomal hydrolases and thus serve as a "receptor".

This approach would seem capable of considerable generalization; most lysosomal enzymes are glycoproteins [27], and thus through an appropriate choice of lectin, it should be possible to promote the uptake of a variety of lysosomal hydrolases, or indeed other glycoprotein enzymes, by cells in culture. This may be of use in developing preliminary approaches to enzyme replacement therapy [28] and may also be of interest in studying the stability, turnover and metabolic functions of exogenous macromolecules in cultured cells.

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