The effect of monensin on β -hexosaminidase transport in normal and I-cell fibroblasts

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The carboxylic ionophore, monensin, blocks the migration of glycoprotein-containing vesicles from the Golgi region to the plasma membrane in fibroblasts resulting in an accumulation of secretory products in the Golgi cisternae. Treatment of cultured I-cell fibroblasts with monensin ($0.5\mu\text{M}$) decreased the abnormal excretion of β -hexosaminidase to 40% of untreated cultures within 15 min. A corresponding intracellular accumulation of the enzyme to >200% of untreated cultures by 24 h was also observed. A small intracellular accumulation and slightly enhanced excretion of β -hexosaminidase occurred in treated normal fibroblast cultures. The intra- and extra-cellular distribution of newly synthesized β -hexosaminidase in both normal and I-cell cultures converged during monensin treatment. β -Hexosaminidase isoenzymes excreted by both monensin-treated normal and I-cell fibroblasts were electrophoretically indistinguishable from the four bands characteristic of I-cell intracellular β -hexosaminidase. The excreted enzyme from both cultures was found to be a low- or no-uptake form. This form of β -hexosaminidase may have been excreted from a secondary route preceding the site of the monensin effect. The similar findings in monensin-treated normal and I-cell cultures suggest that the subcellular site of the biochemical defect in I-cell disease is at a location after the site of the monensin effect i.e. late in the Golgi region or at a post-Golgi-region location.

I-cell disease (mucopolipidosis II) is an autosomal recessive, neurodegenerative disorder, characterized biochemically by an abnormal accumulation of most lysosomal hydrolases in the culture fluid of skin fibroblasts derived from patients (Wiesmann *et al.*, 1971). Unlike hydrolases excreted by normal fibroblasts, I-cell-excreted lysosomal enzymes are not pinocytosed by non-I-cell fibroblasts (Hickman & Neufeld, 1972). This observation led to the hypothesis that in normal fibroblasts, the final packaging of lysosomal enzymes required initially a secretion of the enzymes and then pinocytosis via cell receptors that interacted with a carbohydrate recognition marker on the enzymes (Hickman *et al.*, 1974). It was suggested that in I-cell fibroblasts this marker was absent from the excreted enzymes thus resulting in extracellular accumulation of the enzymes (Hickman & Neufeld, 1972). It was later found that the recognition marker on fibroblast-derived hydrolases is mannose 6-phosphate (Kaplan *et al.*, 1977; Sando & Neufeld, 1977; Ullrich *et al.*, 1978).

We and others have shown, however, that in normal cultures the excretion–re-uptake route only involves 12–15% of the total enzyme when using the

lysosomal hydrolase, β -N-acetyl-D-glucosaminidase (EC 3.2.1.30; β -hexosaminidase) as the prototype (von Figura & Weber, 1978; Vladutiu & Rattazzi, 1979a). We have further shown that the excessive extracellular accumulation of hydrolases in I-cell cultures cannot be accounted for by an impairment of the re-uptake mechanism (Vladutiu & Rattazzi, 1979a). These findings are consistent with our previous proposal that the accumulation of lysosomal enzymes in I-cell culture fluids results from preferential exocytosis (Vladutiu & Rattazzi, 1975). The precise biochemical defect in I-cell disease and its site of action with respect to intracellular hydrolase processing has not been determined.

Monensin is a low-molecular-weight (mol.wt. 680) carboxylic ionophore (Pressman, 1976) with a selective affinity for Na^+ and K^+ , which imposes a high Na^+ and low K^+ concentration in mouse plasma cells and in human fibroblasts and impairs the migration of Golgi-derived vesicles to the cell surface, thus inhibiting the excretion of their contents (Tartakoff & Vassalli, 1977, 1978; Uchida *et al.*, 1979).

The purpose of the present study was to deter-

mine if the incubation of I-cell fibroblast cultures with monensin would impair the excretion of β -hexosaminidase and stimulate the intracellular accumulation of the enzyme, perhaps providing insight into the subcellular location of the biochemical defect in I-cell disease.

Experimental

Chemicals

Monensin was provided through the courtesy of R. L. Hamill of the Lilly Research Laboratories, Eli Lilly and Co., Indianapolis, IN, U.S.A. Mannose 6-phosphate and chloroquine were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Concanavalin A was obtained from Miles Laboratories, Elkhart, IN, U.S.A.

Cell culture

Cultured skin fibroblasts derived from a patient (L. T.) with I-cell disease were obtained from Dr. George Thomas (Johns Hopkins University, Baltimore, MD, U.S.A.). Heterozygote I-cell fibroblasts (GM-81) and normal fibroblasts (GM-41) were obtained from the Genetic Mutant Cell Repository (Camden, NJ, U.S.A.). β -Hexosaminidase-deficient fibroblasts from a patient with Sandhoff disease were obtained from Dr. U. Wiesmann. Homozygous cystic fibrosis fibroblasts were obtained from our own stocks. The cystic-fibrosis cell line was chosen to represent a hereditary disease, which may have an abnormal excretion of hydrolases (Hösli & Vogt, 1977). Fibroblasts were cultured and maintained in 25 cm² plastic flasks and 35 mm Petri dishes (Corning Glass Co., Corning, NY, U.S.A.) with Ham's F-10 medium + 15% foetal calf serum + kanamycin (20 μ g/ml; Grand Island Biologicals, Grand Island, NY, U.S.A.). The collection of fibroblast secretions, the preparations of cell extracts and enzyme assay methods have been described (Vladutiu & Rattazzi, 1975). The fluorogenic substrate, 4-methylumbelliferyl-*N*-acetylglucosamine (Pierce Chemical Co., Rockford, IL, U.S.A.) was used for the assay of β -hexosaminidase and for the detection of electrophoretic patterns (Vladutiu & Rattazzi, 1975). One enzyme unit was defined as the activity that liberated 1 nmol of 4-methylumbelliferone/h at 37°C. Lactate dehydrogenase activity in cell-culture fluids was determined by the method of Wroblewski & LaDue (1955) with a commercial kit (340-UV; Sigma Chemical Co.).

Electrophoresis

Electrophoresis of β -hexosaminidase from normal and I-cell extracts and culture fluids was carried out on cellulose acetate gel (Cellogel; Kalex, Manhasset, NY, U.S.A.) in 36 mM-sodium barbital/citric

acid buffer, pH 6, as previously described (Rattazzi *et al.*, 1975).

Protein

Protein concentrations were measured by using the Coomassie Blue dye-binding method of Bradford (1976).

Monensin effect on β -hexosaminidase distribution

Monolayer cultures of normal, I-cell and cystic-fibrosis fibroblasts were incubated with increasing concentrations of monensin (0.5–4.0 μ M) in serum-free medium containing 0.25% human serum albumin at 37°C for 24 h. The intra- and extra-cellular β -hexosaminidase activity was assayed in each culture at the end of the incubation period. The cytotoxicity of monensin was assessed by quantifying the release of lactate dehydrogenase activity from fibroblasts into the culture fluid.

A time-exposure study of the effect of monensin (0.5 μ M) was carried out on normal, I-cell homozygote and I-cell heterozygote fibroblast cultures. The β -hexosaminidase and lactate dehydrogenase activities were measured in the culture fluids of monolayers treated with monensin for 15 min to 72 h. The intracellular β -hexosaminidase activity was assayed at intervals beginning at 1 h after treatment up to 72 h.

The fraction of newly synthesized β -hexosaminidase (units/ μ g of cell protein) in the intra- and extra-cellular pools was estimated in normal and I-cell cultures treated with 0.5 μ M-monensin for 24 and 48 h. This estimation was based on the results of previous experiments with cycloheximide treatment of normal and I-cell cultures (Vladutiu & Rattazzi, 1979b).

Mannose 6-phosphate-mediated uptake of β -hexosaminidase

We have previously shown that the incubation of normal fibroblast cultures with 2 mM-mannose 6-phosphate for 24 h caused an inhibition of re-uptake of a small but significant portion (2% daily) of high-uptake β -hexosaminidase, which was excreted by the cells (Vladutiu & Rattazzi, 1979a). The effect of monensin (0.5 μ M) on the specific re-uptake of β -hexosaminidase in normal fibroblast cultures by altering the mannose 6-phosphate-recognition marker on the enzyme or the mannose 6-phosphate receptor on the cell membrane was assessed, and the specific effect of monensin on I-cell β -hexosaminidase excretion was determined by incubating monolayers with 1 ml of serum-free medium containing 0.5 μ M-monensin or 2 mM-mannose 6-phosphate or 0.5 μ M-monensin + 2 mM-mannose 6-phosphate/dish at 37°C for 24 h. At the end of the incubation period, the respective cell monolayers and culture

fluids were harvested and assayed for β -hexosaminidase activity.

The effect of monensin on the ability of Sandhoff fibroblasts to pinocytose high-uptake (Glaser *et al.*, 1975) β -hexosaminidase was also determined. Monolayers of Sandhoff fibroblasts were pretreated with 2 ml of either 0.5 μ M-monensin in serum-free medium or serum-free medium alone/dish at 37°C for 24 h. Those cultures pretreated with monensin were then incubated with serum-free medium containing high-uptake β -hexosaminidase + 0.5 μ M-monensin at 37°C for 5 h. The untreated cultures were incubated with high-uptake enzyme alone. The amount of enzyme taken up by the cells was quantified by determining the concentration of β -hexosaminidase per μ g of cell protein (corrected for endogenous enzyme activity). The high-uptake enzyme preparation used for this experiment was collected from the culture fluid of normal fibroblast cultures treated with 25 or 50 μ M-chloroquine + 2 mM-mannose 6-phosphate at 37°C for 18 h (Gonzalez-Noriega *et al.*, 1980). The fluids from several cultures were pooled, concentrated by ultrafiltration at 4°C (membrane XM 100; Amicon Corp., Lexington, MA, U.S.A.) and dialysed overnight at 4°C against 1000 vol. of serum-free medium + Hanks' balanced salt (HBS) solution (1:1, v/v).

The uptake of excreted β -hexosaminidase obtained from normal fibroblasts exposed to 0.5 μ M-monensin was also determined to see if monensin influenced the intracellular processing of β -hexosaminidase, which was destined for excretion, resulting in an abnormal enzyme product. Monolayers of normal fibroblasts were incubated with 0.5 μ M-monensin in serum-free medium at 37°C for 24 h. The culture fluids were harvested, concentrated and dialysed. Monolayers of Sandhoff fibroblasts were preincubated with serum-free medium at 37°C for 24 h and then exposed to the dialysed β -hexosaminidase derived from monensin-treated normal cells for a 5 h uptake period. After uptake, the cells were harvested, extracted and assayed for β -hexosaminidase activity.

A possible direct effect of monensin on high-uptake β -hexosaminidase derived from normal fibroblast culture fluids was also assessed, to determine if pretreatment of the enzyme with monensin altered its specificity for receptor-mediated uptake. A preparation of high-uptake enzyme was collected from normal fibroblasts in the presence of chloroquine + mannose 6-phosphate, then concentrated and dialysed as described above. Monensin was added to the enzyme preparation to a final concentration of 0.5 μ M. As a control, a second portion of the enzyme was mixed with serum-free medium alone. Both preparations were incubated at 37°C for 24 h then dialysed against serum-free medium + HBS

solution overnight. The treated and untreated enzyme preparations were applied to Sandhoff cell monolayers for a 5 h uptake period at 37°C. The cells were harvested, extracted and assayed for β -hexosaminidase activity.

Concanavalin A precipitation

The binding affinity of concanavalin A for mannose residues was used to assess whether any of the β -hexosaminidase isoenzyme excreted from monensin-treated normal and I-cell cultures had any major alterations in their mannose composition. The procedure will be described in detail elsewhere (G. D. Vladutiu & M. C. Rattazzi, unpublished work). Briefly, culture fluids from monensin-treated normal and I-cell cultures and from corresponding untreated cultures were adjusted to a β -hexosaminidase concentration of 500 units/ml and 10 μ l of each was submitted to electrophoresis on cellulose acetate gel. After electrophoresis, each lane was overlaid with 40 μ l of a solution containing 160 μ g of concanavalin A/ml. The gel was then incubated in a moisture chamber at 37°C for 2 h, washed overnight in saline to remove any enzyme not precipitated by concanavalin A and finally stained for β -hexosaminidase activity with the fluorogenic substrate.

Results

Monensin effect on β -hexosaminidase distribution

The effects of increasing concentrations of monensin on the excretion of β -hexosaminidase from normal, I-cell and cystic-fibrosis fibroblasts are presented in Fig. 1. In the I-cell cultures, a progressive inhibition of excretion occurred until excretion was maximally decreased to 40% of the corresponding untreated cultures with 4 μ M-monensin. In normal and cystic-fibrosis cultures, excretion

Fig. 1. Effect of increasing concentrations of monensin on β -hexosaminidase excretion in 24 h

Fibroblast cultures were derived from normal (■), cystic-fibrosis (■) and I-cell-disease (▨) individuals. Results are means for duplicate experiments.

was increased to a maximum of 70 and 40% over untreated cultures respectively with only $0.5\mu\text{M}$ -monensin (Fig. 1). However, normal and cystic-fibrosis cells usually only excrete $\leq 15\%$ of their total cellular β -hexosaminidase in 24 h (Vladutiu & Rattazzi, 1979a); hence, monensin only effected a modest net increase in excretion of 10%. I-cell fibroblasts excrete 85–90% of their total β -hexosaminidase in 24 h (Vladutiu & Rattazzi, 1979a); hence, a 60% decrease in excretion affected 51–54% of the total enzyme.

An enhanced release of lactate dehydrogenase, suggesting a cytotoxic effect of monensin, was apparent only in the cystic-fibrosis cultures, reach-

ing a maximum of 3.8-fold over controls with $4\mu\text{M}$ -monensin.

The time-dependent effect of $0.5\mu\text{M}$ -monensin on the excretion of β -hexosaminidase during the first hour of incubation is presented in Fig. 2. Within 15 min, monensin minimally stimulated excretion of the enzyme in normal cultures but drastically inhibited the excretion in I-cell homozygote cultures.

The effect of $0.5\mu\text{M}$ -monensin on intra- and extra-cellular β -hexosaminidase for a period of up to 48 h is summarized in Table 1. In normal cultures, excretion was maximally affected by 24 h with an additional 8.6% of total cell enzyme being excreted. In contrast, the decrease in excretion in I-cell cultures was maximal at 15 min (Fig. 2) and represented a net 24% decrease in the excretion of total cell enzyme. In cystic-fibrosis cell cultures, excretion was minimally enhanced to 17% over untreated controls by 24 h (results not shown). Monensin minimally affected I-cell heterozygote cultures with respect to excretion until 24 h when values exceeded those obtained in treated normal cultures by 30%.

The intracellular concentration of β -hexosaminidase in normal cells was variable over 48 h with a minimal overall accumulation (Table 1). However, I-cell fibroblasts progressively accumulated enzyme intracellularly to a maximum of $>200\%$ of untreated cultures by 48 h. A small accumulation occurred in heterozygote cells by 24 h ($113 \pm 23\%$) and there was no accumulation in the cystic-fibrosis fibroblasts (98% of untreated value).

In previous experiments, we determined that in cycloheximide-treated I-cell cultures, 40% of the intracellular and 91% of the excreted enzyme was newly synthesized. In similarly treated normal cell cultures, 16% of the intracellular and 5% of the excreted β -hexosaminidase was newly synthesized

Fig. 2. Time-dependent effect of monensin ($0.5\mu\text{M}$) on β -hexosaminidase excretion from normal and I-cell disease fibroblasts

Symbols: Δ and $\blacktriangle$, I-cell disease; $\circ$ and $\bullet$, normal; $\bullet$ and $\blacktriangle$, monensin-treated; $\circ$ and Δ , untreated. Results are means for duplicate experiments.

Table 1. Effect of $0.5\mu\text{M}$ -monensin on intra- and extra-cellular distribution of β -hexosaminidase in normal and I-cell cultures

Time (h)	Normal cultures*				Cell extracts (% of untreated value)	I-Cell cultures*				Cell extracts (% of untreated value)
	Culture fluids			Culture fluids		Cell extracts (% of untreated value)				
	(% of untreated value)	(% of total enzyme value)								
		Monensin	Untreated							
2	92.8 ± 42	2.5	4.0	118 ± 8	42 ± 5	21 ± 4	48 ± 2	124 ± 12		
4	134 ± 67	3.0	2.5	89 ± 8	44 ± 5	31 ± 9	56 ± 8	124 ± 37		
6	122 ± 14	2.0	1.0	101 ± 1	40 ± 4	47	70	100 ± 6		
8	147 ± 13	2.0	1.0	116 ± 7	49 ± 4	38	60	123 ± 15		
24	226 ± 44	21.6 ± 5	13 ± 3	122 ± 36	66 ± 18	59 ± 4	83 ± 3	226 ± 62		
48	210 ± 26	17.0	8.0	109 ± 9	44 ± 17	42	80	230 ± 46		

* Means $\pm$ S.D. for three to eight experiments. S.D. values not included for means of duplicate experiments.

(Vladutiu & Rattazzi, 1979b). The distribution of newly synthesized β -hexosaminidase in normal and I-cell fibroblast cultures treated with monensin for 24 and 48 h is presented in Fig. 3. The concentrations of β -hexosaminidase in both the normal and I-cell culture fluids and in the corresponding extracts begin to converge by 24 h. That is, as β -hexosaminidase excretion is substantially decreased in I-cell cultures, it is increased in normal cultures; an intracellular accumulation of the enzyme occurs in both normal and I-cell fibroblasts. This process, however, is more pronounced and more rapid in I-cell than in normal cells during the first 24 h exposure to monensin.

Mannose 6-phosphate-mediated uptake of β -hexosaminidase

Although monensin slightly enhanced the excretion of β -hexosaminidase in normal fibroblast cul-

tures (Figs. 1–3), this excreted enzyme was not a high-uptake form. This was demonstrated when mannose 6-phosphate failed to enhance the extracellular accumulation of the enzyme in monensin-treated normal cultures above the expected enhanced accumulation (2% of total daily value) in cultures treated only with mannose 6-phosphate (Vladutiu & Rattazzi, 1979a) (Fig. 4), and by the fact that the enzyme derived from monensin-treated normal fibroblasts was not endocytosed by Sandhoff-disease fibroblasts during 5 h uptake experiments (Table 2). However, monensin also had an effect on the mannose 6-phosphate cell receptors and/or internalization of the enzyme as evidenced by a 65% decrease in the ability of monensin-treated Sandhoff fibroblasts to pinocytose high-uptake β -hexosaminidase (Table 2). Hence, the decrease in the extracellular accumulation of β -hexosaminidase in monensin-treated I-cell cultures was not due to a

Fig. 3. Effect of monensin (0.5 μ M) on the intra- and extra-cellular distribution of newly synthesized β -hexosaminidase in normal and I-cell fibroblast cultures during 24 and 48 h exposure

Symbols: ■, I-cell cultures; ▨, normal cultures. C, untreated control; M, monensin-treated. Results are means for two to eight experiments.

Fig. 4. Effect of monensin on the excretion of high-uptake β -hexosaminidase

Cell cultures were incubated with 0.5 μ M-monensin + 2 mM-mannose 6-phosphate (■), 0.5 μ M-monensin (▨), 2 mM-mannose 6-phosphate (■) or with no additions (□). Results are means $\pm$ s.d. for triplicate experiments.

Table 2. Effect of monensin on β -hexosaminidase uptake by Sandhoff fibroblasts

Donor enzyme*	Enzyme applied (units/ μ g of cell protein)	Uptake (units/ μ g of cell protein per h)
Chloroquine (50 μ M) + 2 mM-mannose 6-phosphate	7.8	0.34 $\pm$ 0.01†
	58.7	0.39 $\pm$ 0.01†
Chloroquine (50 μ M) + 2 mM-mannose 6-phosphate	8.5	0.11 $\pm$ 0.01‡
	65.2	0.15 $\pm$ 0.03‡
Monensin (0.50 μ M)	26.6	0.01 $\pm$ 0.01†

* Enzyme collected from normal fibroblast culture fluids in the presence of the compounds described.
† Sandhoff cells preincubated before uptake with serum-free medium for 24 h. Results are means $\pm$ s.d. for triplicate cultures.
‡ Sandhoff cells preincubated with 0.5 μ M-monensin in serum-free medium for 24 h. Results are means $\pm$ s.d. for triplicate cultures.

Fig. 5. Electrophoresis of β -hexosaminidase from cell extracts and culture fluids derived from monensin-treated and untreated normal and I-cell fibroblast cultures. Abbreviations: ICD, I-cell-derived; ext., extract enzyme; med., culture fluid enzyme; mon., monensin; Hex A, β -hexosaminidase A; Hex B, β -hexosaminidase B.

conversion of the excreted enzyme into a high-uptake form resulting in specific re-uptake of the enzyme, because the addition of mannose 6-phosphate to monensin-treated I-cell cultures did not increase the extracellular accumulation of β -hexosaminidase (Fig. 4).

Incubation *in vitro* of high-uptake β -hexosaminidase with $0.5 \mu\text{M}$ -monensin for 5 h followed by dialysis to remove the monensin, did not affect the subsequent uptake of the enzyme by Sandhoff fibroblasts. Hence, there appeared to be no direct effect of monensin on the enzyme itself *in vitro*. An effect on the intracellular processing of β -hexosaminidase, however, resulting in an alteration of the recognition marker on the enzyme was apparent in the production of a 'no-uptake' (Vladutiu & Rattazzi, 1979a) excreted form from monensin-treated normal cells (Fig. 4 and Table 2).

Electrophoresis

Fig. 5 shows the qualitative effects of monensin on β -hexosaminidase in the cells and culture fluids of normal and I-cell fibroblasts. Lanes (1) and (3) show the typical I-cell and normal cell intracellular isoenzyme patterns respectively, which are unaffected by monensin. The typical I-cell-excreted β -hexosaminidase pattern (lane 2) is altered in monensin-treated cultures (lane 5) to the extent that the excreted isoenzyme pattern becomes similar to the I-cell intracellular pattern (lane 1). The normal excreted isoenzyme pattern (lane 4) is also affected by monensin (lane 6), resulting in a pattern that is indistinguishable from the affected I-cell-excreted enzyme (lane 5).

Concanavalin A precipitation

All the β -hexosaminidase isoenzymes in the culture fluids of monensin-treated normal and I-cell fibroblasts were precipitated in cellulose acetate gel after electrophoresis and overlay with concanavalin A, indicating the presence of mannose residues in the carbohydrate composition of the isoenzymes.

Discussion

In I-cell fibroblast cultures, monensin caused a rapid decrease of extracellular β -hexosaminidase with a corresponding intracellular accumulation. In view of the intracellular build-up of β -hexosaminidase in I-cell fibroblasts, it is likely that monensin inhibited the excretion of newly synthesized enzyme resulting in its accumulation presumably in the Golgi region. This finding differs from our previous observations of decreased excretion of β -hexosaminidase induced by tunicamycin, cycloheximide (Vladutiu & Rattazzi, 1979b) or 25°C culture temperature (Vladutiu, 1979), with no intracellular accumulation. These treatments appeared to have inhibited mainly protein synthesis.

The fact that excretion from I-cell fibroblasts was not totally blocked, as evidenced by the excretion of a small amount of the I-cell intracellular-type isoenzymes and that similar isoenzymes were also excreted by monensin-treated normal cells, suggests that two phenomena resulted from monensin treatment: (1) prevention of excretion from a late- or post-Golgi region (mainly evident in I-cell fibroblasts); (2) appearance of a minimal excretion from a secondary pathway in the cells, perhaps from pre-Golgi vesicles (transitional elements from endoplasmic reticulum) in both normal and I-cell fibroblasts. Evidence for pre-Golgi-region vesicles has been established in pancreatic exocrine cells (Jamieson & Palade, 1977) and in Chinese hamster ovary cells (Rothman & Fine, 1980); a direct secretion route via smooth-endoplasmic-reticulum vesicles has been shown in rat liver cells (Morre *et al.*, 1974). It is possible that excretion from this secondary route occurs regularly in normal and I-cell fibroblasts, but is not observable when the more prominent post-Golgi route is functioning, or that it is operative only when there is a back-up of secretory products in the Golgi region.

The I-cell intracellular-type isoenzymes of β -hexosaminidase excreted in both monensin-treated normal and I-cell cultures did not appear to possess the mannose 6-phosphate-recognition marker necessary for uptake by normal or Sandhoff fibroblasts. This supports our contention of pre-Golgi excretion as the enzyme may have been excreted before being fully processed, before either complete glycosylation (Rothman & Fine, 1980) and/or phosphorylation (Hasilik & Neufeld, 1980).

An alteration in glycosylation, either in the intracellular assembly (Vladutiu & Rattazzi, 1975) or in the degradation (Vladutiu, 1978) of carbohydrate chain(s) constituting the mannose 6-phosphate-recognition marker complex, could result in excretion of the I-cell hydrolases as they could no longer be retained in subcellular vesicles on fusion with the plasma membrane (Lloyd, 1977). However, retention of internalized high-uptake β -

glucuronidase in endocytic vacuoles does not require the mannose 6-phosphate-recognition marker (Glaser *et al.*, 1975). It may be that different subcellular vesicles comprising the Golgi-lysosomal-vacuolar system in fibroblasts have different receptor specificities for lysosomal enzymes. In I-cell disease, lysosomal enzymes may enter the excretory vesicles of the system before being fully processed or after being improperly processed with respect to glycosylation (Vladutiu & Rattazzi, 1978).

Hasilik & Neufeld (1980) have suggested that in normal fibroblasts, the phosphorylation of β -hexosaminidase precursors normally occurs at a non-lysosomal site and does not occur at all in I-cell fibroblasts. A defect in either the phosphorylation of hydrolases (Ullrich *et al.*, 1978; Bach *et al.*, 1979) or the specific glycosyl residue to which the phosphate is attached (Bach *et al.*, 1979) has been postulated as the primary defect in I-cell disease. This, however, does not imply re-uptake as a problem in I-cell disease (Vladutiu & Rattazzi, 1979a), but may involve the conversion of a non-secretory product to a secretory one (Sly & Stahl, 1978).

The unaltered electrophoretic pattern of the intracellular β -hexosaminidase in monensin-treated normal cultures is explainable by our contention that only the small fraction of newly synthesized enzyme inside normal cells was affected by monensin (Vladutiu & Rattazzi, 1979b). The bulk of pre-existing enzyme, already packaged in lysosomes, could not have been affected. I-cell fibroblasts, in contrast, doubled their intracellular enzyme concentration in the presence of monensin (Table 1). An electrophoretic change in this accumulated enzyme was not apparent because the effect of monensin resulted in an enzyme form that was electrophoretically indistinguishable from the usual I-cell internal enzyme.

The inability of monensin-treated Sandhoff fibroblasts to pinocytose high-uptake β -hexosaminidase may represent an inhibition of the migration of receptor proteins from the Golgi cisternae to the plasma membrane during membrane biogenesis (Farquhar, 1978).

Our findings with monensin treatment were: (1) that the newly synthesized β -hexosaminidase excreted by both normal and I-cell fibroblasts converged toward the same value by 48 h; (2) that the intracellular build-up of the enzyme in I-cell fibroblasts also approached normal values in 48 h; and (3) that electrophoretically identical isoenzymic forms of β -hexosaminidase were excreted by both normal and I-cell cultures, which all suggest that the subcellular site of the I-cell defect is after the site of the monensin effect i.e. late in the Golgi region or at a post-Golgi location.

Since the intracellular accumulation of β -hexosaminidase in monensin-treated I-cell fibroblasts is

presumably confined to the Golgi region and does not involve lysosomes, monensin is not a 'treatment' for I-cell disease at the lysosomal level in fibroblasts. Our findings, however, contribute to localizing the site of the I-cell defect and to understanding the steps involved in the subcellular processing of lysosomal enzymes.

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