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CORRECTION OF COMBINED β-GALACTOSIDASE/NEURAMINIDASE DEFICIENCY IN HUMAN FIBROBLASTS

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SUMMARY

The combined deficiency of β-galactosidase and neuraminidase in human fibroblasts can be corrected to nearly normal values. This can be accomplished by addition of concentrated culture medium obtained after NH_4Cl stimulation of different types of human fibroblasts, including those with an isolated β-galactosidase or neuraminidase deficiency. The corrective factor is a macromolecular glycoprotein, which is labile at 60°C. Its uptake by human fibroblasts is competitively inhibited by mannose-6-phosphate and its corrective action within β-gal$^-$/neur$^-$ fibroblasts continues during a "chase" of 72 hours.

INTRODUCTION

Various patients with skeletal abnormalities, skin lesions, retinal cherry-red spot, myoclonus and dementia have been found to have a combined deficiency of β-galactosidase and neuraminidase (1-4). The nature of this combined lysosomal enzyme deficiency has not yet been resolved but complementation studies after fusion of different types of human mutant fibroblasts indicate that the responsible gene mutation is different from that involved in the isolated β-galactosidase deficiency in G_{M1}-gangliosidosis (5, 6) and that causing isolated neuraminidase deficiency in mucolipidosis I (4, 7).

When fibroblasts with a combined deficiency of β-galactosidase and neuraminidase (β-gal$^-$/neur$^-$) were co-cultivated with other human fibroblasts, including those with an isolated neuraminidase deficiency (neur$^-$), a slight increase of neuraminidase activity in the β-gal$^-$/neur$^-$ cells was observed (4).

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Preliminary experiments suggested that this partial correction was due to the uptake of a glycoprotein that is secreted by other cells into the culture medium.

The present studies were carried out to obtain more information about the nature of the correction of this combined lysosomal enzyme deficiency.

MATERIALS AND METHODS

Cell cultivation and analysis

Human skin fibroblasts were cultivated in Ham's F10 medium supplemented with 10% fetal calf serum and antibiotics. Fibroblasts from patients with infantile and adult G_{M1}-gangliosidosis were kindly provided by Dr. H. Goldman (Montreal) and Dr. Y. Suzuki (Tokyo) respectively. Cells with an isolated neuraminidase deficiency were derived from a patient with mucolipidosis I (Dr. Bakker, Amsterdam) and two cell strains with a combined deficiency of β-galactosidase and neuraminidase were derived from a 2-year-old boy described by Andria et al. (8) and from an affected 21-week-old fetus described by Kleijer et al. (3). The cells were free of mycoplasm as judged by the method of Chen (9). Enzyme analyses were performed after trypsinization, rinsing in saline, centrifugation (10 min. 100 g) and lysis in double distilled water. The activities of β-galactosidase and neuraminidase were assayed with 4-methylumbelliferyl-β-D-galactopyranoside (Koch-Light) and 4-methylumbelliferyl-α-D-N-acetylneuraminic acid (prepared in Institut für Biochemie II (Med. Fak.), Universität Heidelberg).

To stimulate the secretion of "high-uptake" forms of glycoproteins, fibroblasts were grown to confluency in a 200 cm^2 flask, whereafter the growth medium was replaced by 40 ml of serum free medium to which NH_4Cl was added in a final concentration of 10 mM. After 2 or 3 days the medium was collected, dialyzed against Dulbecco's phosphate buffered saline and concentrated on an Amicon PM 10 filter to a final volume of 1 ml (concentrate). In case cells with normal β-galactosidase activity were used, the β-galactosidase secreted into the medium after NH_4Cl treatment was removed by affinity column chromatography using a p-aminophenylthiogalactoside-CH-sepharose affinity matrix (10).

Correction

To study the corrective effect an aliquot of 40 µl concentrated medium after NH_4Cl treatment was added to 1 ml of Ham's F10 medium supplemented with fetal calf serum and left for a few hours up to three days on different types of mutant fibroblasts.

Correction was also studied by addition of a Concanavalin A preparations of human placenta and liver. Concanavalin A - sepharose 4B (Pharmacia, Uppsala) was used according to a procedure described earlier (11). After elution at 20°C with 0.75 M α-

Table I

EFFECT OF CONCANAVALIN A PREPARATIONS FROM HUMAN PLACENTA AND LIVER ON THE β-GALACTOSIDASE AND NEURAMINIDASE ACTIVITY IN NEURAMINIDASE DEFICIENT FIBROBLASTS*

	ENDOGENOUS ACTIVITY		AFTER ADDITION OF CON.A PREPARATIONS		
	β-gal.	neur.		β-gal.	neur.
β-GAL$^-$/NEUR$^-$ (8)	59.3	1.3	(1)	58.7	3.6
			(2)	67.7	5.9
MUCOLIPIDOSIS I	640	0.4	(2)	650	0.3

*Activities have been measured with 4-methylumbelliferyl substrate and are expressed as 10^{-9}.moles.mg^{-1}.hr^{-1}. (1) represents Con.A preparation from human liver and (2) from placenta.

methylglucoside and dialysis against 10 mM Na-phosphate buffer pH.7 the preparation was concentrated on an Amicon PM 10 filter till a final protein concentration of 15 mg/ml. An aliquot of 50 µl of this preparation was added to 1 ml medium.

Inhibition of β-galactosidase activity

Irreversible inhibition of β-galactosidase activity in cultured human fibroblasts was performed by growing cells for 2 hours in medium containing 0.2 mM β-D-galactopyranosylmethyl-p-nitrophenyltriazene (β-galMNT) kindly provided by Dr. M. Sinnott (School of Chemistry, University of Bristol) according to procedures described earlier (12, 13). After refreshing the medium, the reappearance of β-galactosidase activity was studied during a period of 3 days.

RESULTS

Concanavalin A - sepharose 4B (Con.A) preparations of human placenta and human liver were added for two days to the medium above β-gal$^-$/neur$^-$ fibroblasts and to cells with an isolated neuraminidase deficiency (neur$^-$). The results in Table 1 show a 3 to 5 times increase of the neuraminidase activity in β-gal$^-$/

Table II

EFFECT OF CONCENTRATED "CORRECTIVE FACTOR" ON THE ACTIVITIES OF β-GALACTOSIDASE AND NEURAMINIDASE IN DIFFERENT TYPES OF HUMAN MUTANT FIBROBLASTS*

MUTANT CELL STRAIN	β-GALACTOSIDASE		NEURAMINIDASE	
	endo-genous	after addition "corrective factor"	endo-genous	after addition "corrective factor"
β-GAL$^-$/NEUR$^-$ (8)	64	474	2.2	32
β-GAL$^-$/NEUR$^-$ (3)	80	380	1.0	21
INFANTILE G_{M1}-GANGLIOSIDOSIS	5.5	4.5	90	82
ADULT VARIANT G_{M1}-GANGLIOSIDOSIS (15)	65	55	170	172
MUCOLIPIDOSIS I	610	540	0.5	1.0
CONTROL FIBROBLASTS	350-1050 $\bar{x}$ = 630		43-172 $\bar{x}$ = 82	

*Activities are measured with 4-methylumbelliferyl substrate and are expressed as 10^{-9}moles.mg^{-1}.hr^{-1}. The concentrate of "corrective factor" in these experiments is prepared from medium above G_{M1}-gangliosidosis fibroblasts (infantile type) after NH_4Cl stimulation (see Materials and methods).

neur$^-$ fibroblasts. This increase cannot be due to uptake of neuraminidase, because the activity of neur$^-$ fibroblasts did not increase. Heat inactivation (15' at 100°C) of the Con.A fraction abolishes the partial restoration of the neuraminidase activity of β-gal$^-$/neur$^-$ fibroblasts. These observations suggest that the correction factor is a glycoprotein.

To achieve a more efficient uptake of the correction factor another source of glycoproteins was found in media of various types of human fibroblasts that were treated with NH_4Cl to stimulate the secretion of precursor forms of glycoproteins (14). The medium containing these "high-uptake" forms of glycoproteins was then concentrated and dialyzed on an Amicon pM 10 filter, to remove most of the molecules with a molecular weight lower than 20 x 10^3. The concentrated medium obtained in this way was

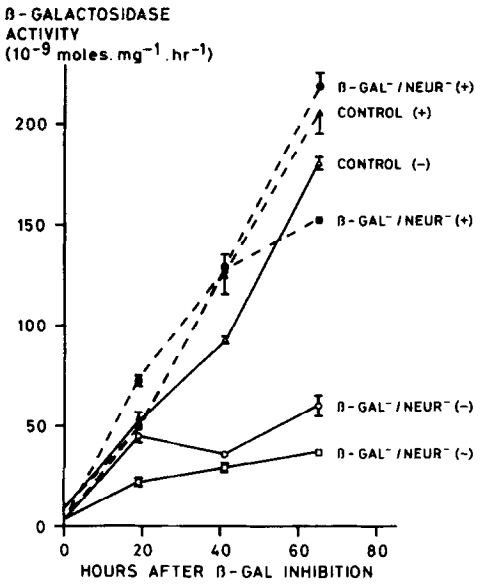

Fig. 1 CORRECTION FOR β-GALACTOSIDASE ACTIVITY IN β-GAL⁻/NEUR⁻ FIBROBLASTS.

After irreversible inhibition of β-galactosidase with a triazene analogue the reappearance of β-galactosidase was studied in two different β-gal⁻/neur⁻ cell strains with and without addition of "corrective factor" prepared from medium above G_{M1}-gangliosidosis fibroblasts after NH_4Cl stimulation.

□ = β-gal⁻/neur⁻ fibroblasts (8)

○ = β-gal⁻/neur⁻ fibroblasts (3)

△ = normal fibroblasts.

Closed symbols represent the activity of β-galactosidase in the same cell strains but after addition of "corrective factor".

added to the culture medium above various types of human mutant fibroblasts and left for 2-3 days. Table II summarizes the effect of concentrate on the β-galactosidase and neuraminidase activity in the different cell types. There was no effect on fibroblasts with an isolated β-galactosidase deficiency (β-gal⁻) nor on neur⁻ cells. In two different strains of β-gal⁻/neur⁻ fibroblasts, however, the activity of both β-galactosidase and neuraminidase increases markedly and approaches low control values. In the experiment illustrated in Table II concentrated medium containing "corrective factor" was prepared from NH_4Cl stimulated medium of G_{M1}-gangliosidosis fibroblasts (β-gal⁻), but similar results were obtained with concentrates from normal and mucolipidosis I media.

Addition of mannose-6-phosphate (1 mM) interferes with the reappearance of β-galactosidase and neuraminidase activities in β-gal$^-$/neur$^-$ cells after addition of "corrective factor". Heat treatment (15 min. at 60°C) of the corrective factor results in a complete loss of its effect, but at 50°C correction is still retained (data not shown).

The reappearance of newly synthesized β-galactosidase molecules after correction of β-gal$^-$/neur$^-$ cells could be studied with the use of an irreversible inhibitor of β-galactosidase (10, 12, 13). Addition of this inhibitor to the medium above β-gal$^-$/neur$^-$ fibroblasts results in a 94% inhibition of the residual β-galactosidase activity. Fig. 1 shows the reappearance of β-galactosidase activity during 65 hours after removal of the inhibitor. In β-gal$^-$/neur$^-$ cells the β-galactosidase activity reaches its original value within 24 hours and subsequently levels off. When a concentrate of "corrective factor" was added, however, the activity of β-galactosidase continues to progress as in control fibroblasts. The reappearance of β-galactosidase activity in control fibroblasts is not affected by addition of "corrective factor".

In subsequent experiments "corrective factor" was added to the medium above β-gal$^-$/neur$^-$ cells during 16 hours followed by a chase of 72 hours (Fig. 2). The intracellular increase of both neuraminidase and β-galactosidase activity was found to continue during 3 days after removal of the "corrective factor" from the medium. In contrast, medium from β-gal$^-$/neur$^-$ fibroblasts, prepared in the same way, had no effect on the intracellular activities of β-galactosidase or neuraminidase in the mutant cells.

DISCUSSION

The complementation for β-galactosidase after fusion of different types of human β-galactosidase deficient cell strains (5) was surprising in view of the fact that normal human β-galactosidase consists of one polypeptide of about 70,000 m.w. which also occurs in an aggregate of molecular weight 700,000 (16). Later, Wenger et al. (1) found a coexistent deficiency of neuraminidase in one of their atypical patients with β-galactosidase deficiency. Fibroblasts from several other patients were sub-

sequently found to have this combined β-galactosidase/neuraminidase deficiency (2, 4). Analysis of interspecies and intraspecies hybrids, Hoeksema et al. (17, 18) showed that the isolated β-galactosidase deficiency in classical forms of G_{M1}-gangliosidosis is due to a structural mutation in the gene on chromosome 3 coding for the β-galactosidase polypeptide chain. In variants, later identified as β-gal$^-$/neur$^-$, the residual β-galactosidase activity consists of the monomeric form of β-galactosidase and aggregation to higher molecular weight forms seems to be impaired.

Using an irreversible inhibitor of β-galactosidase, van Diggelen et al. (10) observed that the turnover time of β-galactosidase in normal human fibroblasts and in G_{M1}-gangliosidosis cells is about 10 days whereas that in β-gal$^-$/neur$^-$ fibroblasts is reduced to less than 1 day. This was found to be due to enhanced degradation of β-galactosidase in these mutant cells (10).

In co-cultivation studies the neuraminidase activity of β-gal$^-$/neur$^-$ cells increased 5-7 fold as a result of transfer of a "corrective factor" secreted by other fibroblasts including those with an isolated neuraminidase deficiency (4). In the present study a similar effect was found after addition of a Con.A preparation of human liver or human placenta to medium above β-gal$^-$/neur$^-$ cells. In both instances only a partial restoration of neuraminidase activity (up to 10-15% of control values) and none of β-galactosidase occurs. When concentrates of culture media from NH_4Cl stimulated fibroblasts are used, however, a complete restoration of the activity of both neuraminidase and β-galactosidase in β-gal$^-$/neur$^-$ cells is achieved. The rate of reappearance of β-galactosidase in β-gal$^-$/neur$^-$ fibroblasts in the presence of corrective factor (Fig. 1) is similar to that in control fibroblasts.

The corrective factor or its precursor form is secreted by various types of human fibroblasts including those from G_{M1}-gangliosidosis (β-gal$^-$) and mucolipidosis I (neur$^-$). No correction is obtained with concentrate prepared from medium after NH_4Cl stimulation of β-gal$^-$/neur$^-$ fibroblasts. This points to the specific nature of the corrective factor. The experimental results presented indicate that the corrective factor is a macromolecular glycoprotein, excluded by an Amicon PM 10 filter, which

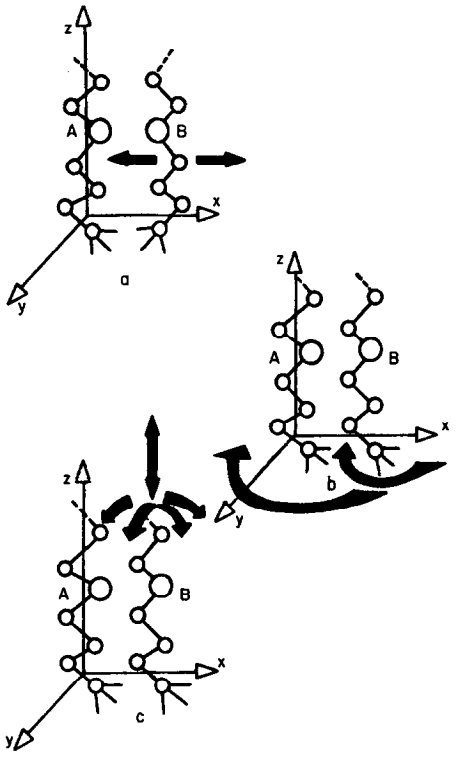

Fig.2 : Schematic presentation of the packing procedure of lipid molecules assembled in monolayers.

b) Monolayer B has the possibility to change its orientation around the Z axis (Fig.3d and Fig.3c). Monolayer A is fixed.

In each case, only the bilayer structure of energy minimum is retained. If each of the structure obtained for the isolated lipid molecule define in table III, were assembled in monolayer (for time consuming reasons we limited our approach to the assembling of 7 molecules) one structure obtained after minimization represents 95% of probability (Table IV). It is obvious that the monolayer packing stabilizes the structure cd. This conformation is characterized by the close proximity of the phosphate residue associated to the hydrophilic moeity of one lipid and the choline residue associated to the adjacent lipid (Fig.4). The electrostatic interaction between the 2 residues stabilizes the lipid structure.

From the structure of the lipid in the monolayer, the organization in the lipid bilayer was theoretically evaluated in an attempt to compare our approach with recent experimental data. Indeed, neutron diffraction combined with the use of selectively deuterated lipids can provide detailed

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