INTERCELLULAR EXCHANGE OF LYSOSOMAL ENZYMES: ENZYME ASSAYS IN SINGLE HUMAN FIBROBLASTS AFTER CO-CULTIVATION

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SUMMARY

Intercellular exchange of N-acetyl-β-D-glucosaminidase (EC 3.2.1.30) β-galactosidase (EC 3.2.1.23) and acid α-glucosidase (EC 3.2.1.20) was studied after co-cultivation of normal and enzyme deficient human fibroblasts in confluent cultures. Enzyme activities were measured in single cells using microchemical procedures. After co-cultivation of normal control fibroblasts and those from a patient with Sandhoff's disease an increase of activity of N-acetyl-β-D-glucosaminidase was found in Sandhoff cells, together with a decrease of activity in normal control cells. After co-cultivation of normal fibroblasts and those from patients with glycogenosis II and GM1-gangliosidosis, no indication was found for intercellular transfer of acid α-glucosidase and β-galactosidase respectively. The significance of the results is discussed in respect of the hypothesis of Hickman and Neufeld about secretion and uptake of lysosomal enzymes.

INTRODUCTION

Cultures of human fibroblasts have proven to be a useful model system for the study of lysosomal storage diseases. Correction of the metabolic defect by administration of enzyme preparations to fibroblasts in culture was reported in several studies (1-7). It has been suggested that enzyme replacement might be a feasible therapy in lysosomal storage diseases.

A more fundamental interest in uptake and secretion of lysosomal enzymes was raised in studies with cultured cells from patients with I-cell disease. For several acid hydrolases increased levels of activity were found in the medium of cultured cells from these patients, together with decreased levels of intracellular activities (8, 9). Hickman and Neufeld suggested that lysosomal enzymes have to be secreted and subsequently are taken up via specific recognition sites in order to reach their

lysosomal destination. I-cell disease would be the result of a defective uptake. To investigate the general validity of this hypothesis we have studied the intercellular exchange of three acid hydrolases in mixed confluent cultures of normal and enzyme deficient cells. In this way administration of enzyme preparations was avoided whereas the conditions for transfer of enzyme were optimal by close contact of donor and acceptor cells. The exchange of N-acetyl-β-D-glucosaminidase (further to be referred as hexosaminidase), β-galactosidase and acid α-glucosidase was studied using fibroblasts from patients with deficiencies for these enzymes as acceptor cells. Enzyme activities were measured in single cells after co-cultivation with normal cells.

METHODS

Cell culture and isolation: Cells were cultured in Ham's F10 medium supplemented with 15% fetal calf serum and antibiotics. In addition to fibroblasts from control subjects fibroblasts were cultured from patients with Sandhoff's disease (deficient in both lysosomal forms of hexosaminidase), GM1-gangliosidosis type 1 (deficient in lysosomal β-galactosidase) and glycogenosis II (deficient in lysosomal α-glucosidase). Preceeding co-cultivation cells from different strains were labeled with either latex or carbon particles. Suspensions of latex particles were obtained from Serva (Dow latex, 0.79 µ) and were added at a concentration of 0.02 ml to 2×10^6 cells in 10 ml culture medium. Suspensions of carbon particles were obtained from George T Gurr (Indian Ink, cat no 51400) and were added at a concentration of 0.1 ml. The medium was removed after one day culturing, the cells were washed three times with 0.9% NaCl and trypsinized. Equal numbers of normal and enzyme deficient cells were mixed and confluent cultures were initiated. Growth in confluency was allowed for one to ten days. The cultures were trypsinized after this period of co-cultivation and reseeded in low density in petri dishes with a thin plastic foil (mylar) bottom, to enable single cell analysis. After cultivation for 20 hrs. differently labeled cells were identified with phase contrast microscopy and localized using a micro grid. Cultures were then freeze dried, whereupon preselected cells could be isolated by microdissection as described by Galjaard et al. (11).

Enzyme assays: 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside, 4-methylumbelliferyl-β-D-galactopyranoside and 4-methylumbelliferyl-α-D-glucopyranoside were used as substrates for the determination of hexosaminidase, β-galactosidase and α-glucosidase activity respectively. Detailed conditions for the assay of β-galactosidase are described by Galjaard et al (12). A method for the single cell analysis of α-glucosidase is described by Reuser et al (13). To assay hexosaminidase activity single fibroblasts were incubated in 0.1 µl phosphate (20 mM)-citrate (10 mM) buffer pH 4.5 containing 5 mM substrate and 0.02%, heat inactivated, bovine serum albumin. The reaction was allowed to proceed for one hour at 37°C and stopped by the addition of 1 µl, 0.5 M carbonate buffer

pH 10.7. Fluorescence was measured in glass capilleries as described for β-galactosidase. Enzyme activities were calculated using a standard curve of methylumbelliferone (MU). Empty pieces of plastic foil were dissected to serve as blanks.

RESULTS

Normal control fibroblasts and enzyme deficient cells were labeled with either latex or carbon particles. Control experiments showed that marker material did not influence the enzyme activities measured. In several experiments equal numbers of fibroblasts from a control subject and from a patient with Sandhoff's disease lacking both lysosomal forms of hexosaminidase were mixed and co-cultivated in confluent cultures for various time periods. As a control both cell lines were cultured separately for the same period. In the experiment described, the cells were co-cultivated for ten days as a confluent culture and then reseeded in low density in petri dishes. After a subsequent cultivation for 20 hours the cells were localized, freeze dried and isolated selectively as described in Methods. Hexosaminidase activity was assayed in single cells of both types.

Fig. 1A shows the frequency distribution of hexosaminidase activity in fibroblasts from control subject and patient when cultured separately. Low residual activity was present in Sandhoff cells (mean activity 0.06×10^{-12} moles MU per hr) whereas in normal fibroblasts the mean activity was 4.6×10^{-12} moles MU per hr. After ten days of co-cultivation marked changes were noticed in the distributions as shown in Fig. 1B. The mean activity of Sandhoff cells was increased to 0.7×10^{-12} moles MU per hr whereas the mean activity of control cells was decreased to 1.7×10^{-12} moles MU per hr. The same effect was found after three days of co-cultivation although less prominent.

In similar experiments exchange of β-galactosidase was studied. Fibroblasts from a patient with GM1-gangliosidosis, lacking lysosomal β-galactosidase, were co-cultivated with normal human fibroblasts in equal numbers for eight days. The frequency distributions of enzyme activity for both types of cells when cultured separately are

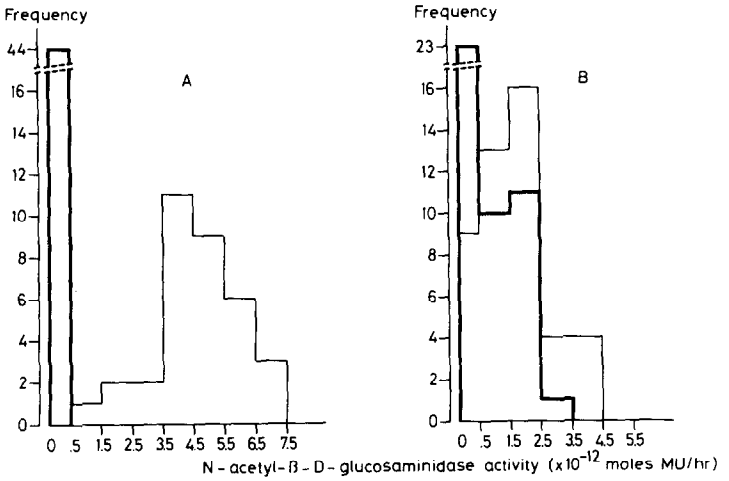

Fig. 1. Frequency distributions of hexosaminidase activity in single human fibroblasts derived from a control subject and from a patient with Sandhoff's disease.
A. Separate cultivation
B. Co-cultivation for 10 days in confluency
▬ patient — control

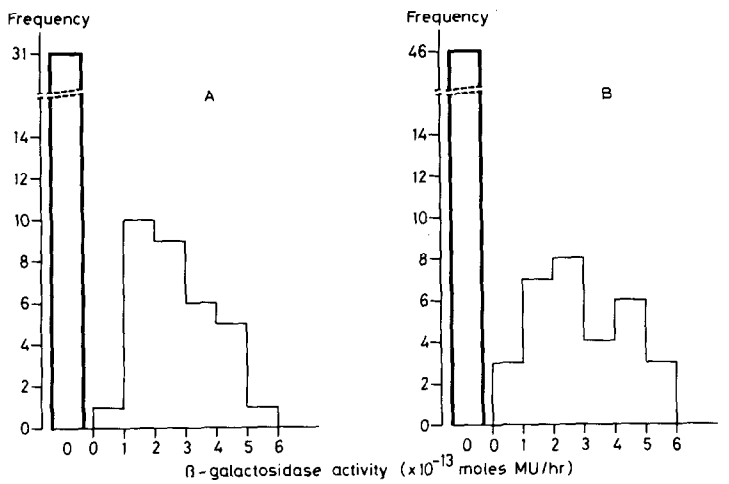

Fig. 2. Frequency distributions of β-galactosidase activity in single human fibroblasts derived from a control subject and from a patient with GM1 gangliosidosis.
A. Separate cultivation
B. Co-cultivation for 8 days in confluency
▬ patient — control

given in Fig. 2A. Distributions after co-cultivation are shown in Fig. 2B. In neither case β-galactosidase activity could be demonstrated in fibroblasts from the patient. The mean activity of normal cells was 1.36×10^{-13} and 1.38×10^{-13} moles MU per hr. respectively. This indicates that no intercellular exchange of β-galactosidase occurred.

Fibroblasts from a patient with glycogenosis II lacking acid α-glucosidase were used as acceptor cells to study exchange of this enzyme. The experiments were done in a similar way as described above. Cells were co-cultivated for seven days. The frequency distributions of α-glucosidase activity of normal and deficient cells in separate and mixed cultures are shown in Fig. 3A and 3B respectively.

No enzyme activity could be demonstrated in fibroblasts from the patient with glycogenosis II. The mean activity of normal cells was 4.7×10^{-14} moles MU per hr. when cultured alone and 3.9×10^{-14} after co-cultivation.

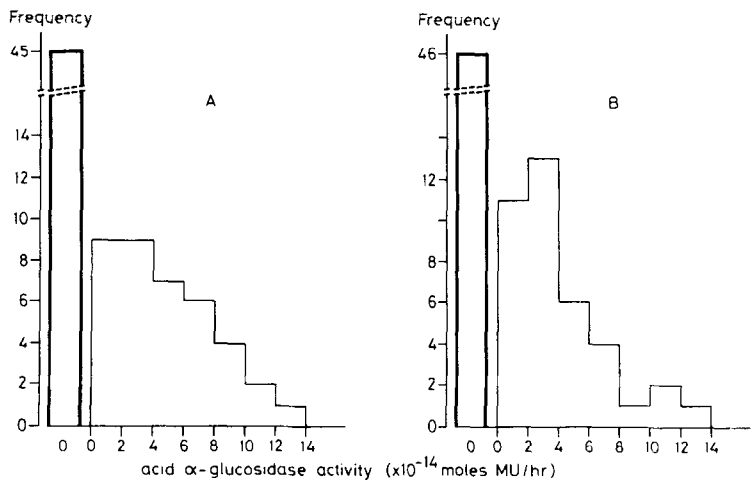

Fig. 3. Frequency distributions of acid α-glucosidase activity in single human fibroblasts, derived from a control subject and from a patient with glycogenosis II.
A. Separate cultivation
B. Co-cultivation for 7 days in confluency
▬ patient — control

No transfer of acid α-glucosidase activity from normal to deficient cells was found.

DISCUSSION

Selective and bulk pinocytosis are distinguished for uptake of extracellular material. The former process requires specific recognition sites on the cell surface as well as on the molecule that is taken up (14). According to the hypothesis of Hickman and Neufeld (10) secretion followed by selective uptake is essential for packaging of lysosomal enzymes. This implies the possibility of transfer of enzyme from one cell to another. Mutual correction of the metabolic defect in cultured fibroblasts from clinically different patients with mucopolysaccharidosis was demonstrated to be the result of free exchange of lysosomal enzymes (1, 2, 3). The hypothesis of Hickman and Neufeld is mainly based on studies with I-cells and the authors indicate that the theory might not apply to all lysosomal enzymes. Strong evidence has been presented for the uptake of hexosaminidase via specific recognition sites (15).

We have studied exchange of hexosaminidase, β-galactosidase and α-glucosidase by co-cultivation of normal control and enzyme deficient cells in confluent cultures thereby trying to approach the in vivo situation, avoiding the administration of enzymes in quantities far exceeding the intracellular activity.

The clear transfer of hexosaminidase activity from normal cells to enzyme deficient cells fits in with the Hickman and Neufeld hypothesis. Interestingly the increase in hexosaminidase activity in Sandhoff cells, as a result of co-cultivation, is accompanied by a loss of activity in normal fibroblasts. No compensation seems to occur for the loss of intracellular activity from normal cells.

No transfer of β-galactosidase or α-glucosidase activity could be demonstrated in our experiments. This might indicate that for these enzymes the packaging into lysosomes is different compared to hexosaminidase. In this case the simultaneous lack of β-galactosidase and hexosaminidase in I-cell disease can not be explained by a mutation affecting recognition sites on the enzyme molecules.

A different explanation for our results might be that the enzyme deficiencies in GM1-gangliosidosis and glycogenosis II are caused by a defect in the recognition sites on the cell surface, preventing uptake of β-galactosidase and α-glucosidase respectively. No cross-reacting material could be demonstrated in liver cells of a patient with glycogenosis II (16) which may support this hypothesis. Detection of cross-reacting material in the liver cells of a patient with GM1-gangliosidosis (17) however, points to a structural mutation in the enzyme molecule rather than to a defect in the uptake of β-galactosidase.

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