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CHARACTERIZATION OF ENZYMATIC DEFICIENCIES OF BRANCHED CHAIN AMINO-ACID CATABOLISM IN HUMAN FIBROBLASTS BY GENETIC COMPLEMENTATION

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Leucine and Isoleucine metabolism in cultured skin fibroblasts from patients with leucinosis, β-Ketothiolase deficiency, propionic, methylmalonic and isovaleric acidemia, was compared with that in normal fibroblasts.

A simple assay was developped using ($U^{14}C$) Isoleucine and ($U^{14}C$) Leucine as substrates. Radioactive incorporation into protein aminoacids were measured. The ($U^{14}C$) branched chain aminoacid incorporation into proteins provides an estimation of the protein synthesis and the incorporation ratio (^{14}C) Aspartate + (^{14}C) Glutamate / (^{14}C) branched chain aminoacid, measures the integrity of the metabolic patnway. Complementation tests permits to characterize the genetic defect.

The incorporation ratio was significantly decreased in blocked pathways, namely in leucinosis and isovaleric acidemia in the presence of ($U^{14}C$) Leucine and in Leucinosis, β-Ketothiolase deficiency, propionic and methylmalonic acidemia in the presence of ($U^{14}C$) Isoleucine. There was a significant restoration of activity in mutant strains with distinct genetic defects after polyethylene-glycol fusion. This assay provides a valuable tool to screen for enzymatic deficiencies of branched chain aminoacid catabolism.

Branched chain aminoacid catabolism is one of the metabolic fields in which genetic disorders are the most comon. More than twelve defective steps have been described to date (1,2). Some of them disclosed genetic and biochemical heterogeneity, i.e. methylmalonyl-CoA mutase (3) and propionyl-CoA carboxylase (4). Other defective steps will be probably discovered in the future. Culture skin fibroblasts are now widely used to investigate these disorders. Direct enzymatic assays are usually necessary to ascertain the diagnosis. Nevertheless some difficulties may arise in the presence of crude tissue preparation and so many deficiencies are present that it may be not possible to perform all of them. Several methods for a rapid delineation of deficient enzyme activity in a pathway have been proposed : $^{14}CO_2$ production from labelled branched chain aminoacids (5) or radioactive incorpora-

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tion from (^{14}C) organic acids into trichloracetic acid precipitable material (6). Nevertheless they usually explore a small part of a metabolic pathway and they may require high amount of cells and difficult CO_2 collection procedure. The purpose of this work was to develop a simple and fast assay which provides a definite diagnosis of defects of branched chain aminoacid catabolism.

This was realized by measuring radioactive incorporation into aspartic and glutamic acid from hydrolyzed proteins, after incubation of the cells with ($U^{14}C$) Leucine or Isoleucine. The rationale for the hydrolyzation of cell proteins is as follows : during the incubation, labelled carbons from Leucine or Isoleucine are incorporated via Krebs cycle into Aspartic and Glutamic acid. Concomitantly aminoacids are incorporated into proteins. The (^{14}C) Leucine or Isoleucine incorporation into proteins provides an estimation of the protein synthesis and the cell viability and the ratio (^{14}C) Aspartate + (^{14}C) Glutamate / (^{14}C) branched chain amino acid indicates the ability of cells to transform (^{14}C) branched chain amino acid into (^{14}C) Aspartate and (^{14}C) Glutamate which are then incorporated into proteins. This transformation needs the integrity of the branched chain aminoacid catabolic pathway.

To further characterize the defects, complementation tests after polyethylene-glycol fusion have been performed between defective cell lines and strains with known defects of the same metabolic pathway. The same methodology as above was used to appreciate the restoration of activity. The absence of complementation between a defective cell line and one previously characterized strain means that both have the same genetic deficiency. This was concomitantly confirmed by the ability of this cell line to complement all other mutant strains in this pathway.

MATERIAL AND METHODS

Twelve mutant strains and six controls have been used throughout this study. Mutant strains included 3 leucinosis, 3 isovaleric acidemia, 1 β Ketothiolase deficiency, 2 propionic acidemia and 3 methylmalonic acidemia. Diagnosis were made clinically and biologically in patients with leucinosis. All of them were of the classical type (7). Other patients have been previously reported (8, 9, 10). Complementation groups in strains with propionyl-CoA carboxylase or methylmalonic-CoA mutase deficiencies have been kindly determined by L.E. ROSENBERG, Yale USA.
Fibroblasts were grown in RPMI 1640 medium supplemented with 10% fetal calf serum in 25cm^2 Falcon flasks. When confluent, mono-

layers of fibroblasts were washed twice with phosphate buffered saline. 1ml of RPMI 1640 medium supplemented with 10% fetal calf serum and 1mM of ($U^{14}C$) Leucine or Isoleucine (1 µCi/ µmole) was added. After 24 h of incubation at 37°C the flasks were washed two times with phosphate buffered saline and the cells trypsinized. Proteins were precipitated with 5 % cold trichloracetic acid and washed two times with 5 % cold trichloracetic acid. After centrifugation 200 µl of 6N HCl was added to the pellet ; the suspension was sealed under nitrogen and incubated at 110°C for 24h. Then the solution was lyophylized. 100 µl of 0.1 N HCl was added and an aliquot was applied to a Technicon aminoacid analyzer. 1 ml fractions were collected and counted by a liquid scintillation spectrometer after adding 10 ml of scintillation cocktail GP, Packard.
Complementation was performed by mixing equal amounts of two different strains in a 25 cm^2 Falcon flask. After a few hours cells were washed three times. Polyethylene glycol (PEG 1000 Merck) 50 % in RPMI 1640 was then added for 20 sec. and after thorough washing the cells were incubated with RPMI 1640 and 20 % fetal calf serum. Using a microscope with phase contrast we have checked that more than 50 % of the cells are hetero karyons. After 24 h (^{14}C) Leucine or Isoleucine was added and the experiment performed as described above.
L ($U^{14}C$) Leucine and L ($U^{14}C$) Isoleucine were purchased from Amersham, England. For purification, portions of 1 ml (50 µCi) of the aqueous solution acidified with 0.3 ml of IM HCl were applied to 0.9 x 0.4 cm carbon exchange column of Biorad AG 50W x 4 H+ form 210-400 mesh (Biorad Lab RIchmond, Ca). The column was washed with 30 ml of water and ($U^{14}C$) Leucine or Isoleucine was eluted with 15 ml of 1 M HCl. After taking to dryness in a rotary evaporator, the residue was dissolved in phosphate buffered saline. Other products were from Sigma.

RESULTS

Using normal skin fibroblasts, after hydrolyzation of cell proteins and ion exchange resin column chromatography, three radioactive peaks appeared. They coeluted with authentic unlabelled Aspartic acid , Glutamic acid, and Leucine or Isoleucine respectively. There was no labelling in other aminoacids and the basal line was near zero. (^{14}C) incorporation into protein aminoacids was linear up to 72h and with respect to the number of cells (up to 10^6 cells).

In the presence of ($U^{14}C$) Leucine the (^{14}C) Aspartate + (^{14}C) Glutamate / (^{14}C) Leucine incorporation ratio was significantly diminished ($p < 0.01$) in all mutant strains except β-ketothiolase deficient cell line (see the figure 1). The decreased incorporation of Leucine-derived products in leucinosis and isovaleric acidemia was consistent with the postulated metabolic block, namely a defect in the conversion of leucine to acetyl-CoA. In propionic and methylmalonic acidemia, although the metabolic pathway was theorically normal, (^{14}C) incorporation was significantly decreased but to a less extent than in both other mutations.

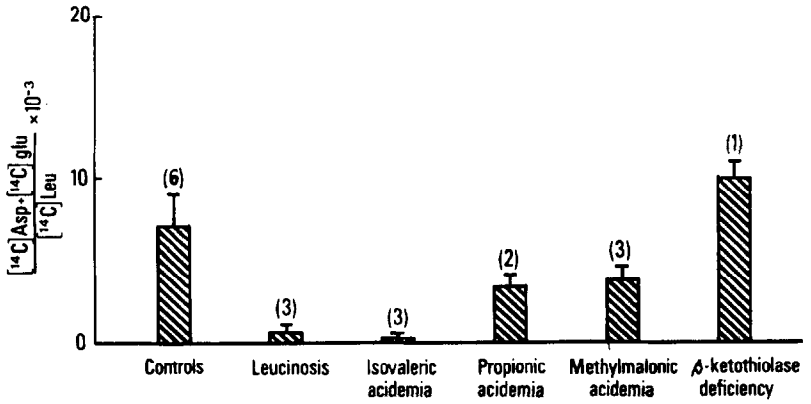

Fig. 1 (^{14}C) Aspartate + (^{14}C) Glutamate / (U^{14}C) Leucine incorporation ratios in normal and mutant fibroblasts after incubation for 24 h in the presence of 1 mM (U^{14}C) Leucine (1µCi/µmle).
(number of strains in parenthesis ; mean ± SD).

In the presence of (U^{14}C) Isoleucine the (^{14}C) Aspartate + (^{14}C) Glutamate / (^{14}C) Isoleucine incorporation ratio was significantly decreased ($p < 0.01$) in all mutant strains (see the figure 2) compared with the normal cell lines.

Studies of the (^{14}C) Glutamate / (^{14}C) Aspartate incorporation ratio into cell proteins from either (U^{14}C) Leucine or (U^{14}C) Isoleucine in normal fibroblasts was most interesting. Indeed,

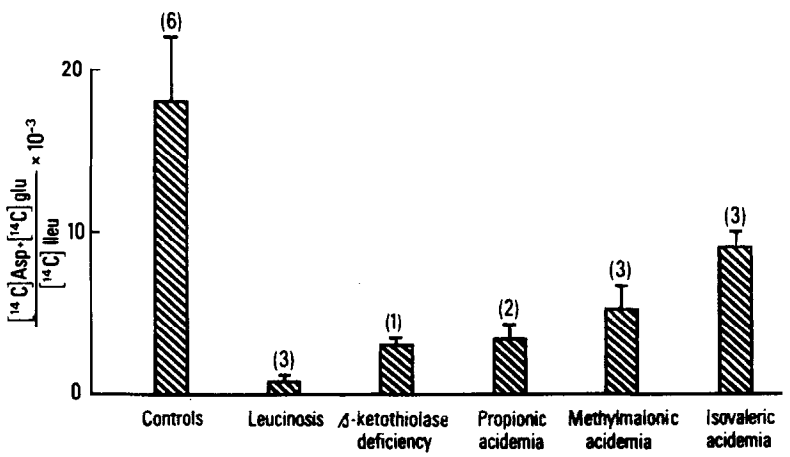

Fig.2 (^{14}C) Aspartate + (^{14}C) Glutamate / (U^{14}C) Isoleucine incorporation ratios in normal and mutant fibroblasts after incubation for 24h in presence of 1 mM (U^{14}C) Isoleucine (1µCi/µmle).
(number of strains in parenthesis ; mean ± SD)

TABLE 1 - (^{14}C) Glutamate/ (^{14}C) Aspartate incorporation ratios in normal and mutant fibroblasts.

Cell Lines	(U^{14}C) Leucine	(U^{14}C) Isoleucine
Controls (6)	2.5 ± 0.2	0.8 ± 0.1
Propionic acidemia (2)	2.2 ± 0.1	2.1 ± 0.1
Methylmalonic acidemia (3)	2.3 ± 0.2	2.1 ± 0.2
Isovaleric acidemia (3)	2.2 ± 0.1	1.2 ± 0.1
Leucinosis (3)	2.2 ± 0.3	1.1 ± 0.2
β-Ketothiolase deficiency (1)	2.6 ± 0.3	1.3 ± 0.2

Fibroblasts were incubated for 24h in the presence of 1mM (U^{14}C) Isoleucine or 1mM (U^{14}C) Leucine.

in the presence of (U^{14}C) Isoleucine the ratio was 2.5 ± 0.2 (n = 6) and 0.8 ± 0.1 (n = 6) in the presence of (U^{14}C) Leucine (see the table 1). The ratio was not significantly modified in mutant strains except in strains from patients with methylmalonic and propionic acidemia in which the ratio in presence of (U^{14}C) Isoleucine became similar to this in presence of (U^{14}C) Leucine.

In complementation studies polyethylene-glycol fusion between strains with isovaleric acidemia and leucinosis was able to restore the (^{14}C) Aspartate + (^{14}C) Glutamate / (^{14}C) Leucine incorporation ratio (see the table 2). Similarly in the presence of (U^{14}C)

TABLE II - Complementation test between strains from patients with leucinosis and isovaleric acidemia.

	Leucinosis	Isovaleric acidemia
leucinosis	< 1	6.3
isovaleric acidemia		< 1

Results are expressed as the magnification of the ratio (^{14}C) Aspartate + (^{14}C) Glutamate / (^{14}C) Leucine incorporated into protein after polyethylene glycol treatment as compared to coculture. Incubation conditions as in table I. The substrate was 1mM (U^{14}C) Leucine. All tests were done in duplicate.

TABLE III - Complementation tests between strains from patients with leucinosis, β Ketothiolase deficiency, propionic and methylmalonic acidemia.

		Methylmalonic Acidemia			Propionic Acidemia		β-Ketothiolase deficiency
		MUT-	CblA	CblB	A	BC	
Methylmalonic Acidemia	MUT-	<1	2.2	8.7	2.2	3.2	7.1
	CblA		< 1	1.7	1.5	2	2.7
	CblB			< 1	3.8	6.1	9.3
Propionic Acidemia	A				< 1	1.6	3.4
	BC					< 1	1.9
β-Ketothiolase deficiency							< 1

Expression of the results and methodology as in Table 2 except the substrate which was 1 mM ($U^{14}C$) Isoleucine. Complementation groups among cell lines from patients with methylmalonic and propionic acidemia are designated as reported by Rosenberg L.E. (2).

Isoleucine complementation by polyethylene glycol fusion was positive between each different genetic abnormalities (see table 3). In constrast, polyethylene glycol fusion between equal amounts of two cell lines belonging to the same genetic group never restore this ratio.

DISCUSSION

The decreased radioactive incorporation into Aspartic and Glutamic acid in mutant cell lines is consistant with the hypothesis that our assay is a valuable tool to screen for the enzymatic deficiencies of branched chain aminoacid catabolism. The partial depression of incorporation either in strains with defective propionyl-CoA carboxylase and methylmalonyl-CoA mutase in the presence of ($U^{14}C$) Leucine or in strains with isovaleric acidemia in the presence of ($U^{14}C$) Isoleucine was unexpected. However, it has been shown that in patients with these organic acidemia numerous intermediates accumulated above the metabolic block (11). Thus it is more likely that the inhibition of Leucine or Isoleucine oxidation was secondary caused by a metabolic effect such as accumulated metabolites which increase because of the primary enzyme deficiency. Indeed, it has been reported that isovaleryl-CoA inhibited decarboxylation of all three branched chain amino-

acid (12). In contrast a residual incorporation such as observed in strains totally defective in propionic and methylmalonic acidemia was expected because in these defects one of both products, namely acetyl-CoA, may enter freely the Krebs cycle and therefore may be incorporated into Aspartic and Glutamic acid.

The striking difference that we observed in Glutamate/Aspartate ratio values between incubations in the presence of Leucine and Isoleucine was most interesting. It probably arises from that products are different. Leucine is metabolized to acetyl-CoA and Isoleucine to acetyl-CoA and propionyl-CoA. Indeed in propionic and methylmalonic acidemia in which propionyl-CoA is not further metabolized, an identical Glutamate/Aspartate ratio was observed in the presence of Isoleucine or Leucine.

The method described here provides information under quasi physiological conditions about net metabolic activity of branched chain aminoacid catabolism pathways. Coupled with complementation tests it permits to specify which particular enzyme is deficient and moreover to study the eventual genetic heterogeneity of an enzymatic defect. Problems concerning control of cell number are avoided and the incubation time may be easily increased as far as necessary, particularly if number of cells are small since fibroblasts are incubated in the presence of usual medium supplemented with fetal calf serum and under aseptic conditions. This method may be applied to amniotic cells, leukocytes or platelets and we are thinking it will serve most effectively for the investigation of many disorders of other aminoacids catabolism in intact cells.

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