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Research Article

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Complementation Studies of Isovaleric Acidemia and Glutaric Aciduria Type II Using Cultured Skin Fibroblasts

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ABSTRACT Using cultured skin fibroblasts, we studied the heterogeneity of inborn errors of leucine metabolism such as isovaleric acidemia (IVA), glutaric aciduria type II (GA II), and multiple carboxylase deficiency (MC). We first developed a simple macromolecular-labeling test to measure the ability of cells to oxidize [1-^{14}C]isovaleric acid in situ in culture. Cells from two different lines were fused using polyethylene glycol, and the ability of the heterokaryons to oxidize [1-^{14}C]isovaleric acid was tested by the macromolecular-labeling test. The MC line complemented with all 10 IVA lines tested; heterokaryons showed 99±68% more activity than the unfused mixture of component cells. GA II/IVA heterokaryons exhibited poor growth, but when the culture remained confluent, the GA II cells complemented with all six IVA lines tested, showing a 71±41% increase in activity. The relatively large standard deviations are due to a few experiments in which significant enhancement of macromolecular-labeling test activity was not observed upon fusion, but significant complementation was clearly observed in repeats of the same combinations. These results are consistent with our previous findings, which indicated that the decreased ability of GA II cells to oxidize isovaleryl-CoA involves a defective electron-transporting system rather than a defective isovaleryl-CoA dehydrogenase. IVA/IVA heterokaryons showed no complementation in any combination tested, indicating no detectable heterogeneity in isovaleric acidemia. This finding indicates that the same gene is mutated in all IVA lines. Previous results indicated that this gene codes for isovaleryl-CoA dehydrogenase.

INTRODUCTION

Several inborn errors involving leucine catabolism have been identified (1). One of these is isovaleric acidemia (IVA),[1] in which the genetic defect occurs at the level of dehydrogenation of isovaleryl-CoA. This disease is clinically characterized by increased levels of isovaleric acid in the blood (2, 3) and by excessive urinary excretion of isovalerylglycine (4). Episodic accumulation of isovaleric acid accompanied by severe vomiting, lethargy, coma, and ketoacidosis occurs in patients suffering from this inherited disease. Based on the pattern of metabolite accumulation (2, 4) and on in vitro studies of substrate oxidation using cells from IVA patients (5), a genetic mutation of a substrate-specific isovaleryl-CoA dehydrogenase in patients with IVA had been postulated. In fact, Rhead and Tanaka (6) recently demonstrated a specific deficiency of mitochondrial isovaleryl-CoA dehydrogenase activity in skin fibroblasts from patients with IVA using a tritium release assay devised for this purpose. In contrast, mitochondrial butyryl-CoA dehydrogenase activity in IVA cells was maintained at normal levels. These recent studies have elucidated the biochemical mechanisms of IVA. IVA appears to be transmitted by an autosomal recessive inheritance. This conclusion is supported by the occurrence in multiple sibs in many families, and approximately equal incidence in both sexes (1). Parents are phenotypically normal but the ability of their cells to oxidize [2-^{14}C]leucine is ~50% of normal values, indicating heterozygosity (5).

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[1] *Abbreviations used in this paper:* EMA, ethylmalonic-adipic aciduria; GA II, glutaric aciduria type II; IVA, isovaleric acidemia; M, mild form of IVA; MC, multiple carboxylase deficiency; MEM, minimal essential medium; MLT, macromolecular-labeling test; MSUD, maple syrup urine disease; PEG, polyethylene glycol; S, severe form of IVA.

The question of possible heterogeneity in IVA has frequently been raised from clinical standpoint because some patients exhibit severe acidosis immediately after birth (7–9), while others have relatively mild symptoms that manifest themselves only later in childhood by mild acidosis (2, 3, 10, 11). However, it is not yet known whether such variation in clinical expression is due to genetically determined heterogeneity or not.

Leucine metabolism is also blocked at the level of isovaleryl-CoA dehydrogenation in another inborn metabolic disorder, glutaric aciduria type II (GA II) (12–15). Like IVA, this disease is characterized by the excretion of large amounts of isovaleric acid, but unlike patients with IVA, several other short-chain fatty acids and dicarboxylic acids including glutaric, adipic, ethylmalonic, isobutyric, and 2-methylbutyric acids are excreted as well (12, 13, 15). Thus, the defect in GA II comprises a wide range of metabolic reactions in which several different short-chain acyl-CoA, including isovaleryl-CoA, are dehydrogenated. From these observations it has been postulated that GA II is not due to a mutation of the various acyl-CoA dehydrogenases per se, but rather to a deficiency of one of the proteins involved in the transfer of electrons from acyl-CoA dehydrogenases to coenzyme Q of the mitochondrial electron transport chain (12–14). Recent studies by Rhead et al. (14) and those by Goodman et al. (13) have shown that three acyl-CoA dehydrogenases (*n*-butyryl-CoA, isovaleryl-CoA, and glutaryl-CoA dehydrogenases) were in fact normal in GA II fibroblasts. Ethylmalonic-adipic aciduria (EMA) (16) is due to a similar mechanism, but its clinical manifestations are less severe than those of GA II.

To determine if there is any detectable genetic heterogeneity in IVA and to demonstrate that the defect in GA II involves a gene which is different from that for isovaleryl-CoA dehydrogenase, we developed a simple macromolecular-labeling test (MLT), which permitted us to undertake an extensive survey of the activity of enzymes involved in the oxidation of leucine after the production of isovaleryl-CoA. With this method we tested the ability of cultured skin fibroblasts obtained from patients with blocks in leucine catabolism to oxidize [1-^{14}C]isovaleric acid. Furthermore, we tested heterokaryons produced by fusion of pairwise combinations of various mutant cell lines for their ability to oxidize [1-^{14}C]isovalerate.

METHODS

Materials. Two batches of [1-^{14}C]isovaleric acid (10 mCi/mmol) were synthesized by New England Nuclear (Boston, MA).

Cell lines. Cell lines were derived from 9 normal individuals, 12 IVA patients, 1 GA II patient, 1 EMA patient, and 1 biotin-responsive multiple carboxylase deficiency (MC) patient (17, 18) (Table I), and one maple syrup urine disease (MSUD) patient. IVA cell lines were divided into two groups, severe (S) and mild (M) types, according to the severity of clinical manifestations of the patient from whom the individual cell line was derived. Patients who had severe acidotic episodes leading to lethargy and coma in the first 2 wk of life and their siblings were classified as the severe type while others, who did not have such life-threatening episodes in this period, were classified as the mild type (1). Clinical and biochemical characterization of most of these patients except two IVA patients, have been reported elsewhere as indicated in Table I.

Cell culture. Human skin fibroblasts were grown in modified Eagle's minimal essential medium (MEM; Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal calf serum, nonessential amino acids, and kanamycin. The effect of avidin was studied by using cells grown for one passage in supplemented media containing 1.2 mg avidin (Sigma Chemical Co., St. Louis, MO) per liter of media. Harvesting was done with 0.25% trypsin-EDTA (Gibco Laboratories).

MLT for isovalerate oxidation. The MLT for isovalerate oxidation was similar to the method used by Willard et al. (19) to test the ability of cells to oxidize propionate. It is based on the following principles: in a normal cell, isovaleric acid labeled with a radioactive carbon at the carboxyl group is metabolized to unlabeled acetoacetic acid and labeled acetyl-CoA (Fig. 1). When the labeled acetyl-CoA enters the tricarboxylic acid cycle, some of the radioactive label will appear in glutamic and aspartic acids via transamination of α-ketoglutaric and oxaloacetic acids, respectively (19, 20). The labeled acetyl-CoA can also be used in the biosynthesis of fatty acids and cholesterol. All of these products can be

TABLE I
Identification of Cell Lines

Cell line	Cell type and reference	
262	IVA-M	(11)
501	IVA-S	(23)
502*	IVA-M	(2, 3, 5)
503*	IVA-M	(2, 3, 5)
642	IVA-M	(10)
743	IVA-M	(26)
747	IVA-S	(24)
763‡	IVA-S	
765§	IVA-S	(9)
766§	IVA-S	(9)
778‖	IVA-M	
834	IVA-S	(25)
493	EMA	(16)
605	GAII	(12)
741	MC	(17, 18)

* 502 and 503 cell lines are from two siblings.
‡ 763 cell line was donated by Dr. David Valle, Johns Hopkins University School of Medicine, Baltimore, MD.
§ 765 and 766 cell lines are from two siblings.
‖ 778 cell line was donated by Dr. W. Zipf, Columbus Children's Hospital, Columbus, OH.

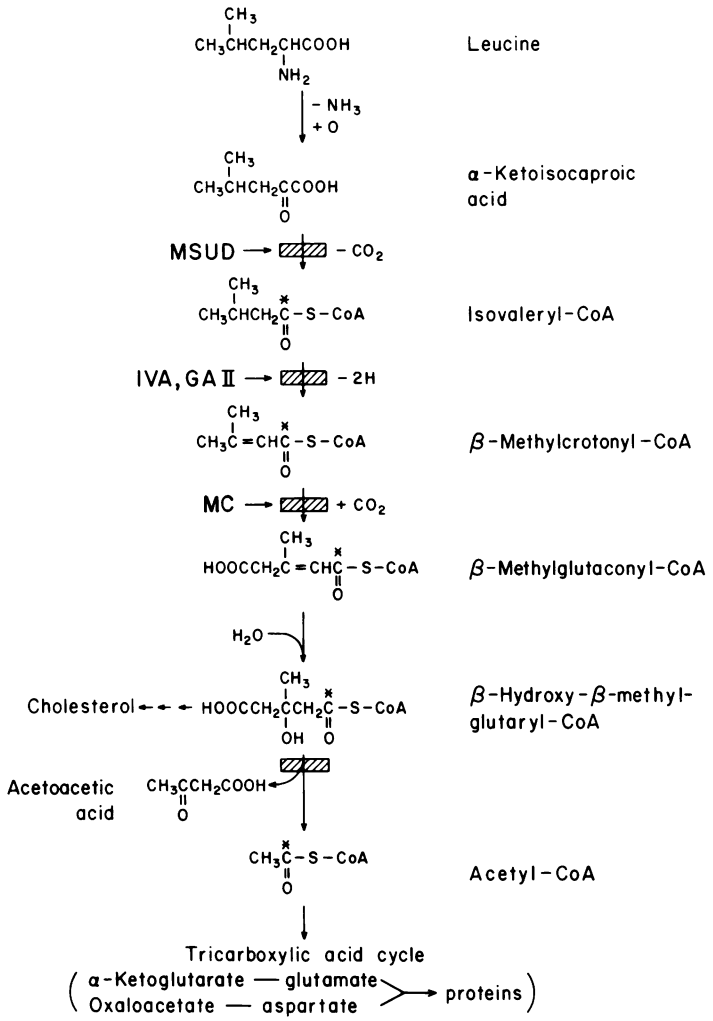

FIGURE 1 The pathway of leucine metabolism and the fate of ^{14}C from [1-^{14}C]isovaleric acid. The radioactive carbon is marked with an asterisk. The metabolic blocks in MSUD, IVA, and MC are indicated by shaded rectangles.

incorporated into macromolecules that are precipitable by addition of trichloroacetic acid (TCA). Radioactivity will be present in the TCA precipitate of cells, which can oxidize leucine from the point of isovaleryl-CoA production. However, cells with a block in leucine catabolism after the formation of isovaleryl-CoA will show a decreased amount of ^{14}C incorporation into TCA-precipitable material.

To perform the MLT, duplicate (or triplicate) tissue culture dishes (60 × 15 mm, Costar Data Packaging, Cambridge, MA) were seeded with 5×10^5 cells/plate in supplemented MEM. The next day the medium was removed and 1.5 ml of a reaction mixture consisting of the standard medium supplemented with 50 mM glucose, and [1-^{14}C]isovalerate sodium salt (0.5 mCi/mmol; 1 mM final concentration; 0.75 μCi/plates; New England Nuclear) was applied to each dish. After incubating at 37°C for 2 d, the reaction mixture was aspirated and the cells were washed with phosphate-buffered saline (PBS: pH 7.4), harvested with 0.25% trypsin-EDTA, collected in cold (0°–4°C) PBS and centrifuged at 4°C at 300 *g* for 20 min. The supernatant was aspirated and the cell pellet resuspended in 1.5 ml cold deionized water. To this suspension a 50% TCA solution was added to make a 10% final TCA concentration. A 10-min precipitation on ice followed. TCA-precipitable material was pelleted by centrifugation at 300 *g* for 5 min. The precipitate was solubilized in 0.5 ml 1 N NaOH and an aliquot was counted in Biofluor. The remainder of the sample was neutralized with HCl and used to determine protein concentration by the method of Lowry et al. (21).

In the course of this study, two different batches of [1-^{14}C]isovaleric acid produced by custom synthesis by New England Nuclear were used as substrate. The first batch was sealed in several ampules and stored for 10 yr before use. When we switched to the new batch in the course of this study, we noted that the results of MLT obtained using the new substrate were approximately twice as high as those obtained with the old [1-^{14}C]isovaleric acid, even though the

specific activity was adjusted as described in the preceeding section, but the power of segregating the normal and various mutant cell lines remained unchanged. Therefore, we carried out MLT on six normal cell lines using the old or new substrates and determined ratios of the two values. The range of these ratios in different cell lines were close, averaging 2.09±0.31. The values obtained using the old [1-^{14}C]isovaleric acid were then multiplied with this coefficient and combined to those obtained using the new [1-^{14}C]isovaleric acid.

Cell fusion. For complementation analysis, equal numbers of cells from two cell lines were mixed before plating. Four tissue culture dishes were seeded with 1×10^6 cells/plate. The next day, or after the dishes reached confluency, the medium was removed from two dishes and the monolayers were treated with a 50% (wt/vol) solution of autoclaved polyethylene glycol 6000 (PEG; J. T. Baker Chemical CO., Phillipsburg, NJ) in serum-free MEM for 60 s. After the PEG solution was removed, calcium-free medium (MEM suspension culture) was then applied to aid in reducing PEG toxicity (22). The two remaining dishes were not treated with PEG but otherwise treated similarly. The two PEG-untreated plates served as controls. After 15 min the calcium-free medium was removed and 5 ml supplemented medium was added. Unless otherwise mentioned, cells were then incubated at 37°C for 16 h and at the end of this period reaction mixture for MLT was added and a 24-h incubation followed. Only the plates, which were near confluent, were harvested.

RESULTS

Oxidation of [1-^{14}C]isovalerate by normal and mutant cells. The mean ability of each cell type to oxidize [1-^{14}C]isovalerate as measured by MLT is summarized in Table II. IVA lines exhibited a severely decreased ability to oxidize [1-^{14}C]isovalerate. Values for the 12 lines ranged from 1.9 to 3.7 nmol with a mean value of 2.7 nmol ^{14}C incorporated/mg protein per d. This represents 13% of control. Variations in incorporation values between the 12 lines were similar to the variations that were observed within each line.

TABLE II
Results of [1-^{14}C]Isovalerate-MLT on Normal and Mutant Cells

Cell line	Number of cell lines	Number of determinations per cell line	Activity
			nmol incorporated/mg protein/d
Control	9	2–7	20.5±4.0
IVA	12	2–9	2.7±0.6*
GA II			
Severe case	1	8	4.8±1.3*
EMA	1	6	16.5±3.1
MC	1		
Normal media		8	7.3±2.9‡
Avidin media§		2	0.5, 1.9

Data are presented as mean±SD.
* Significantly different from control ($P < 0.001$) as tested by the t test.
‡ Significantly different from control ($0.001 < P < 0.01$) as tested by the t test.
§ Avidin 1.2 μg/ml MEM.

Cells from a patient with the most severe form of GA II (No. 605) exhibited a low level of activity, which was 20% of control. An EMA cell (No. 493) line with a milder deficiency had activity of 16.5 nmol ^{14}C incorporated/mg protein per d, slightly lower, but not statistically different from controls. The ability of these lines to produce $^{14}CO_2$ from [2-^{14}C]leucine was also compared with control cells in a previous study (16). As with the MLT method, line No. 493 exhibited a much higher activity than did line No. 605, producing 23% as much CO_2 as controls, while line No. 605 produced only 9% as much CO_2 as controls. Both cell lines could be readily detected as mutants with $^{14}CO_2$ evolution method. Thus, the MLT can accurately identify cells with severe metabolic blocks, but it lacks the sensitivity necessary to detect cells that are only mildly affected.

The MC line grown in the standard medium also showed a decreased ability to oxidize [1-^{14}C]isovalerate. The value was sufficiently low to enable us to use this line as a positive control in fusions with IVA cells in complementation studies, but in an effort to lower the activity of these cells, we tested the effect of avidin to exhaust the availability of biotin in culture media, since these cells were derived from a biotin-responsive patient. As can be seen in Table II, ^{14}C incorporation dropped to 16% of the mean value obtained using the usual media. This is 5% of the mean of control values. In contrast, the amount of ^{14}C incorporated by cells from two IVA lines grown in avidin-containing media dropped to 65% of the usual mean value, while normal cells from three lines grown in avidin media incorporated 72–92% the usual amount of ^{14}C (data not shown).

We also tested the ability of cells from a patient with MSUD to oxidize [1-^{14}C]isovalerate. In this disease the block in leucine catabolism occurs at the step immediately preceding the dehydrogenation of isovaleryl-CoA (Fig. 1). This cell line showed normal activity and incorporated 35.1 nmol ^{14}C/mg protein per d.

Complementation analysis. Normal/normal combinations: To test the effects of PEG treatment alone, five normal/normal cell combinations were fused and their ability to incorporate ^{14}C from [1-^{14}C]isovalerate was tested. PEG treatment generally caused a 0–47% decrease in the incorporation of ^{14}C. A slight increase of 2% was noted for one combination (Table III).

IVA/MC combinations: IVA/MC combinations served as a positive control to ascertain that the PEG-

TABLE III
Effects of PEG Treatment on [1-^{14}C]Isovalerate-MLT in Normal Cells

Normal cell lines		Change of activity
		% change
82	86	−47
82	87	+2
82	105	−40
86	87	−11
86	105	0

induced cell fusion was producing heterokaryons and that the MLT was sensitive enough to detect complementation in the fused cells. To determine the optimum conditions required for complementation, a time-course experiment was conducted in which MLT reaction mixture was applied to the cells at various times after PEG fusion. Complementation could be detected as early as 6 h after PEG fusion and plateaued at 12–48 h. For most of the data in Table IV, the reaction mixture was added to the cells 16–24 h after fusions and the cells were harvested 24 h after the reaction mixture addition (Methods).

We next tested the possibility of making the enhancement of activity in IVA/MC heterokaryons more pronounced by lowering the base-line activity of the MC line by growing and fusing the cells in avidin-containing media. The two IVA/MC combinations complemented, but growth in avidin-containing media did not lead to enhancement of heterokaryon activity to values significantly greater than that observed when cells were grown in the normal media.

Table IV summarizes the results of complementation tests for MC cells and 10 IVA lines. More than 87% of IVA/MC hybrid cultures reached near-confluence. Complementation in IVA/MC heterokaryons was observed for all 10 IVA lines. In 17 of 19 experiments, there was a 17–280% increase in the amount of ^{14}C incorporated by fused cells than by the same combination of unfused cells. The amount of increase in ^{14}C incorporation varied, perhaps reflecting a differing degree of viability of the fused cells, as well as the proportion of true heterokaryons formed. In 2 of the 19 experiments performed with the IVA lines, only an insignificant increase (4%) in ^{14}C incorporation occurred after fusion with the MC line. Each of these two experiments involved two different IVA cell lines. A significant enhancement of activity was observed with fusion in the repeat experiment of the same MC/IVA combinations. These results indicated that heterokaryons were in fact being formed by the fusion method and that the MLT could detect complementation.

TABLE IV
Complementation for IVA/MC Combinations

		MLT activity		
MC cell	IVA cell	Fused cells	Unfused cells	Change with fusion
		nmol/mg protein/d		*%*
774	501	10.95	6.26	75
		12.80	4.29	198
		5.76	3.01	91
744	502	7.38	3.98	85
		10.80	3.88	178
744	503	9.79	5.70	75
		3.11	1.40	122
744	262	8.81	5.17	70
744	642	7.43	3.80	96
744	743	6.90	5.47	26
		8.99	3.80	137
744	747	4.41	2.42	82
		7.19	6.90	4
744	763	7.54	1.98	280
		5.49	5.28	4
744	765	7.38	2.98	148
		8.31	3.47	139
744	766	6.92	4.28	62
		2.34	2.00	17
Mean±SD		7.50±2.83*	4.00±1.48	99.4±68.4

* Significantly different from the mean value for unfused cells as tested by the *t* test ($P < 0.001$).

IVA/IVA combinations: 86% of IVA/IVA hybrid cultures reached near-confluency. The results of complementation are presented in Table V. We tested 51 different IVA/IVA combinations: 17 of them were S/S, 11 were M/M, and 23 were S/M combinations. Since all of the combinations except two were tested at least twice, the total number of tests was 116 including 42, 21, and 53 for S/S, M/M, and S/M combinations, respectively. Most of IVA/IVA combinations showed a decrease in ^{14}C incorporation after fusion, as in the cases of normal/normal fusion. In 20 cases there was a slight increase in activity, but these increases were not nearly as great as those seen in IVA/MC combination, none exceeding 15%. They more likely represent experimental variation rather than true complementation, since self-fusion of two lines (No. 501 and 502) also exhibited this kind of slight increase. These small positive values occurred 6 times

TABLE V
Complementation for IVA/IVA Combination

IVA(S) cells	IVA(S) cells	MLT activity: Fused cells	MLT activity: Unfused cells	Change with fusion	IVA(S) cells	IVA(S) cells	MLT activity: Fused cells	MLT activity: Unfused cells	Change with fusion
		nmol/mg protein/d		%			*nmol/mg protein/d*		%
A. Combination of two severe phenotypes									
501	501	5.51	5.78	−5	763	766	1.78	1.63	9
		6.77	6.18	10			2.37	2.45	−3
501	747	1.52	2.06	−26	763	834	1.42	1.64	−13
		2.64	3.52	−25			2.19	2.67	−18
501	763	3.03	2.99	1			1.41	1.93	−27
		1.80	2.08	−13					
501	765	2.15	2.49	−14	765	765	1.77	2.27	−22
		3.59	3.90	−8			2.58	3.19	−19
		1.69	2.19	−23			1.81	1.99	−9
501	766	2.93	3.21	−9	765	766	2.09	3.47	−40
		4.04	3.95	3			3.38	4.17	−19
		2.24	2.81	−20			1.71	2.11	−19
747	747	2.72	3.12	−13	765	834	1.82	2.27	−20
		2.31	2.68	−14			1.25	1.62	−23
747	763	1.72	1.70	1			1.62	2.17	−25
		2.10	2.58	−19					
747	765	1.79	2.08	−14	766	766	1.42	1.55	−8
		1.19	1.90	−37			0.99	1.03	−4
747	766	3.04	3.56	−15	766	834	1.76	1.69	4
		1.29	1.42	−9			0.99	1.46	−32
763	765	2.31	2.55	−9			1.21	1.24	−2
		1.79	2.92	−39					
		1.83	2.40	−24	Mean±SD		2.20±1.15	2.49±1.20	−14.5±12.1

IVA(M) cells	IVA(M) cells	MLT activity: Fused cells	MLT activity: Unfused cells	Change with fusion	IVA(M) cells	IVA(M) cells	MLT activity: Fused cells	MLT activity: Unfused cells	Change with fusion
		nmol/mg protein/d		%			*nmol/mg protein/d*		%
B. Combination of two mild phenotypes									
502	502	2.94	2.82	4	262	743	1.34	1.86	−28
		1.87	2.09	−11			3.05	3.29	−7
502	262	3.47	3.43	1	262	778	1.12	1.40	−20
		2.15	2.19	−2			1.69	1.95	−13
502	743	1.54	2.08	−26	642	743	1.94	2.34	−17
		2.99	3.82	−22					
503	262	2.06	2.37	−13	743	778	0.96	1.46	−34
		3.08	3.93	−22			1.01	1.30	−22
							1.29	1.42	−9
262	262	1.34	1.50	−11					
		1.74	2.04	−15	778	778	0.96	1.21	−21
262	642	1.42	1.68	−15					
		1.97	2.37	−17	Mean±SD		1.90±0.78	2.07±0.76	−15.2±9.5

TABLE V (*Continued*)

IVA(S)	IVA(M)	MLT activity: Fused cells	MLT activity: Unfused cells	Change with fusion	IVA(S)	IVA(M)	MLT activity: Fused cells	MLT activity: Unfused cells	Change with fusion
		nmol/mg protein/d		%			*nmol/mg protein/d*		%
C. Combination of a severe and a mild phenotypes									
501	502	1.50	2.39	−37	763	778	1.40	1.75	−34
		3.99	4.27	−7			1.36	1.72	−21
501	503	2.10	3.38	−38	765	503	2.32	2.95	−21
		5.30	4.59	15			3.13	3.33	−6
501	262	5.00	4.68	7	765	262	2.19	2.27	−4
		3.84	3.86	−0.5			3.24	3.65	−11
		3.19	2.83	13					
501	642	1.88	2.00	−6	765	743	1.68	2.21	−24
		1.97	1.83	8			2.20	2.96	−26
							1.64	2.71	−39
501	743	1.18	2.32	−50					
		4.22	4.10	3	765	778	1.50	1.87	−20
							1.14	1.45	−21
747	502	2.40	3.42	−30			0.87	1.40	−38
		1.67	1.68	−0.6					
747	503	1.83	1.82	0.5	766	503	2.21	2.52	−12
		1.31	1.14	15			1.81	3.41	−52
747	642	1.14	1.00	14	766	262	1.88	2.01	−6
		1.47	1.35	9			3.63	3.80	−4
747	743	0.94	1.79	−47	766	743	3.81	4.53	−16
		3.17	2.97	7			1.45	1.80	−19
		1.76	2.37	−26					
763	262	2.67	3.74	−29	766	778	1.65	1.79	−8
		4.74	4.12	14			0.97	1.21	−20
		2.34	2.70	−13					
		2.70	2.85	−5	834	262	1.41	1.87	−25
							1.58	1.61	−2
763	503	2.55	3.06	−17					
		2.35	2.65	−11	834	743	1.45	1.77	−18
							1.19	1.81	−34
763	743	4.63	4.45	4					
		3.98	4.01	−1					
		2.59	3.34	−22	Mean±SD		2.26±1.15	2.66±1.03	−13.4±17.6

in 42 tests in S/S combination, 2 times in 21 tests in M/M combination, and 12 times in 53 tests in S/M combination. Mean values for MLT activity for fused cells were 2.20±1.25 nmol/mg protein per d for S/S combination, 1.90±0.78 nmol/mg protein per d for M/M combination, and 2.26±1.15 nmol/mg protein per d for M/S combination. Neither the frequency of positive percent change nor the mean MLT activity of the S/M group were significantly different statistically from those for the S/S and M/M groups as tested by the chi-square test.

As with IVA/MC combinations, we tested to see if longer periods of incubation after fusion and after the reaction mixture addition would result in enhancement of activity. Although experiments with incubation periods of up to 48 h after fusion and of up to 48 h after the reaction mixture addition were performed, no complementation among any of the IVA lines was observed.

IVA/GA II combinations: Although the GA II line, No. 605, was difficult to grow since it did not survive >3 d after reaching confluency, we attempted fusions of IVA and No. 605 cells. In some experiments the IVA/GA II cultures did not survive well after PEG treatment. 58% of those that survived after PEG treatment reached near confluency and could be further

TABLE VI
Complementation for GA II/IVA Combinations

GA II cell	IVA cell	MLT activity: Fused cells	MLT activity: Unfused cells	Change with fusion
		nmol/mg protein/d		%
605	743	7.19	2.77	160
605	262	11.66	6.61	76
605	763	9.66	5.30	82
		4.99	4.63	8
605	503	4.02	2.95	36
		5.89	3.90	51
605	501	8.46	5.25	61
605	747	8.88	4.56	95
Mean±SD		7.59±2.39*	4.50±1.19	71.1±42.3

* Significantly different from the mean value for unfused cells as tested by the *t* test ($0.001 < P < 0.01$).

tested by MLT. Despite the difficulties encountered with growing IVA/GA II heterokaryons, cultures that remained in good condition after fusion, with a single exception, exhibited significant increases in ^{14}C incorporation, ranging from 36 to 160% (Table VI).

DISCUSSION

We devised a simple assay method, MLT, which can detect cells with severe blocks in leucine catabolism occurring after the formation of isovaleryl-CoA. Using this MLT, the values of ^{14}C incorporation for 12 IVA lines averaged ~13% of the amount of ^{14}C incorporated by normal cells, with a small range of variation. This finding suggests that although the cells were obtained from clinically different IVA patients, the degree of isovaleryl-CoA dehydrogenase deficiency in these different IVA cells is similar. Other cell types with severe metabolic blocks, including MC and GA II cells, also showed a decreased ability to incorporate ^{14}C. The activity of MSUD cells as measured by MLT was normal, as expected from the known metabolic block in this disease. EMA fibroblasts, however, had values close to the lower limit of the normal, although they were consistently lower than controls.

For complementation analysis, combinations of IVA and MC cells were first tested. Complementation was invariably observed with all IVA/MC combinations, indicating that the fusion method was producing heterokaryons and that the MLT was capable of detecting the increased activity of complementing cells.

We next investigated the possible heterogeneity in IVA by testing for complementation in fused cultures of 12 different IVA cell lines. These IVA cells were derived from patients with different clinical symptoms: some experienced severe vomiting and coma only a few days after birth (9, 23–25), and others manifested symptoms of IVA later in childhood (10, 11, 19, 26). Despite the large number of cell lines tested and the clinical heterogeneity of the patients from whom they were derived, we did not observe clear evidence for complementation in any combinations, including those of severe and mild types. These findings indicate that there was no intergenic complementation in the 12 IVA cell lines and suggest that the same gene coding for isovaleryl-CoA dehydrogenase is mutated in all IVA cells.

The finding that there is no detectable intergenic complementation in IVA is consistent with our recent characterization of the isovaleryl-CoA dehydrogenase purified from rat liver mitochondria. This enzyme has been completely separated from four other acyl-CoA dehydrogenases, including butyryl-CoA dehydrogenase, by a sequence of several chromatographic procedures (27, 28). This enzyme is composed of four identical subunits of 43,000 D (28). In other diseases, where clear genetic complementation has been previously found, such as propionic acidemia, methylmalonic acidemia, and MSUD, the enzymes involved are known to have multiple-component enzymes (29), nonidentical subunits (30–32), or require a specific organic cofactor that must be biosynthesized (33, 34). Thus, the genetic heterogeneity in these diseases detected by complementation tests similar to ours are mostly due to mutation of genes encoding for different peptides.

When we compare the values in heterologous IVA (S/M) combinations with those in homologous (S/S or M/M) combinations, the mean MLT activities in fused and unfused cells were essentially identical. However, the frequency of small positive changes in the activity in fused cells was slightly greater (12/53) in the heterologous (S/M) combination group than in the homologous (S/S and M/M) combination group (8/63), although this difference was not statistically significant as tested by the chi-square test. Particularly, in 501/262, 747/503, and 747/642 combinations, positive changes were observed in more than one experiment, raising a possibility of intragenic complementation. To check the possibility that longer incubations would result in IVA/IVA complementation, we utilized the varied incubation period after fusion and after the reaction mixture addition. Although some fused cell combinations were incubated for 4 d before harvest, clear complementation was still not observed. We may conclude at present that there is no detectable com-

plementation in the IVA cell lines tested under the present conditions. However, there is still a possibility that the isovaleryl-CoA dehydrogenase gene is mutated at different positions in different IVA cells. Intragenic complementation involving mutants with different allele of the same gene would require more sensitive method for detection.

Lastly, we examined IVA/GA II cell combinations. Although the GA II lines were difficult to grow, there was large enhancement of activity in fused cells in cultures that remained in good condition. This demonstrates that although the same step in leucine catabolism is blocked in both IVA and GA II, these two diseases are due to different mechanisms. This is consistent with the previous biochemical findings from this and other laboratories, which showed that the acyl-CoA dehydrogenases are normal in GA II cells (13, 14). A more likely mechanism of this disease involves a defect in one of the electron transfer proteins, such as electron transfer flavoprotein, or electron transfer flavoprotein dehydrogenase, which carries electrons from the acyl-CoA dehydrogenase to coenzyme Q in the main mitochondrial electron transport chain (13, 14, 16).

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