

Glutaric Acidemia Type II: Heterogeneity of Clinical and Biochemical Phenotypes¹

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ABSTRACT. We have examined 23 fibroblast lines from patients with neonatal and late onset glutaric acidemia type II and fibroblasts from four parents of these patients. Fifteen of these patients are previously unreported. Results of these investigations show deficiency of electron transfer flavoprotein or electron transfer flavoprotein-ubiquinone oxidoreductase activity in all of the patients' fibroblasts. Immunoblots indicate that the steady state levels of the antigens is very low or undetectable in most of the neonatal onset patients; however, cross-reacting antigen without electron transfer activity is observed in several glutaric acidemia type II fibroblast lines. Assay of parental lines confirm the autosomal transmission of deficiencies of proteins. Of particular interest is the clinical heterogeneity among these patients. Patients may present with an extrapyramidal movement disorder as observed in glutaric aciduria type I, without the typical organic aciduria typical of glutaric acidemia type II even in the presence of severe enzyme deficiency, or with renal cystic dysplasia accompanying electron transfer flavoprotein deficiency. Renal cystic dysplasia had previously been reported only in patients with electron transfer flavoprotein-ubiquinone oxidoreductase deficiency. (*Pediatr Res* 27: 311-315, 1990)

Abbreviations

GA2, glutaric acidemia type II
ETF, electron transfer flavoprotein
ETF-QO, electron transfer flavoprotein-ubiquinone oxidoreductase
MCAD, medium chain (general) acyl-CoA dehydrogenase
Q₁, 2,3-dimethoxy-5-methyl-6-isopentenyl-1,4-benzoquinone

GA2 is an inborn error of fatty and amino acid metabolism characterized by hypoketotic hypoglycemia, metabolic acidosis, and the urinary excretion of substrates, or the metabolites of substrates, of mitochondrial flavoprotein dehydrogenases that transfer electrons to ubiquinone via ETF and ETF-QO. In some patients, the disease is due to deficiency of ETF, a soluble flavin adenine dinucleotide-containing heterodimer located in the mitochondrial matrix (1-5), and in others the disease is due to a deficiency of ETF-QO, an iron-sulfur flavoprotein located in the inner mitochondrial membrane (3, 6-8).

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Patients who develop symptoms during the first few days of life usually do not survive for more than a few weeks, and many of them show anomalies such as renal cystic dysplasia, facial dysmorphism, rocker bottom feet, and abnormalities of the external genitalia (9). Patients with milder forms of the disease may develop episodes of hypoketotic hypoglycemia, acidosis, and hepatomegaly in infancy or childhood, or may present even later with muscle weakness and carnitine deficiency (10-12). The purpose of this report is to describe a new catalytic assay for ETF, and the clinical features and catalytic and immunologic properties of the two electron transferases in 23 patients with severe and mild forms of GA2. Fifteen of these patients have not been previously reported. The results show that (1) renal cystic dysplasia may occur in ETF deficiency, but less frequently than in ETF-QO deficiency, (2) as with glutaric acidemia type I, the disease may present with extrapyramidal movement disorder due to degeneration of the basal ganglia, and (3) urine organic acids may be normal even in patients with severe enzymatic deficiency. One patient was found with an apparent primary defect in the smaller, or β -ETF subunit. As expected, we also found that mutations of ETF and ETF-QO can affect activity without altering protein size or reactivity with polyclonal antibodies. The data also provide additional evidence that ETF and ETF-QO deficiencies are inherited as autosomal recessive traits.

MATERIALS AND METHODS

Patient material. Cultured fibroblasts were grown in Eagle's minimal essential medium containing Earle's salts, 10% fetal bovine serum, antibiotics, and nonessential amino acids. Patients from whom fibroblast lines 1441 (13), 1196 (14), 1728 (15), 1902 (16), 1730 (17), 1808 (18), 1691 (3, 8), 1591 (10), and 1592 (11) were derived have been described. Those patients from whom the other cell lines were obtained have not been reported, and brief descriptions of these patients follow.

Line 1692 was from the fetal sibling of a girl who had died at 3 d of age with hypoglycemia, acidosis, and an acrid odor, and whose autopsy showed fatty degeneration of heart, liver, and renal tubular epithelium, and dilatation of the renal collecting tubules. Large quantities of glutaric acid were detected in the liver. A diagnosis of GA2 was suggested by increased (2.6 $\mu\text{g}/\text{mL}$) glutaric acid in amniotic fluid and, upon termination of pregnancy, fetal autopsy showed large quantities of fat in the liver. The couple has since had another pregnancy in which glutaric acid was not increased in amniotic fluid, and which resulted in the birth of an unaffected child.

Line 1803 was from the aborted sibling of a girl who had died at 3 d of age with hypothermia and apnea, with postmortem findings of microvesicular lipid in heart, liver, and large amounts of glutaric acid in liver and kidney. Pregnancy was terminated when glutaric acid was found to be increased in amniotic fluid at 15 wk; the fetus showed no gross abnormalities. Glutaric acid

was not increased in the amniotic fluid of a later pregnancy, which resulted in the birth of an unaffected infant.

Line 1846 was from a female infant who developed hypoglycemia, lethargy, tachypnea, and metabolic acidosis during the first hours of life, and died 4 d later. Glutaric acid, sarcosine, and other compounds typical of GA2 were detected in urine, which also contained pipecolic acid. Autopsy showed fatty changes in liver parenchyma, cardiac fibers, and cells of the proximal renal tubules. There was no renal cystic dysplasia. Lines 1847 and 1848 are from the mother and father of this child.

Line 1851 was from a boy who at 10 mo of age showed failure to thrive, hypotonia, and gross motor delays, with episodes of ketotic hypoglycemia associated with infections. Urine organic analysis showed increased glutaric, 5-hydroxyhexanoic, adipic, suberic, and sebatic acids, and on another occasion, only increased adipic, suberic, and sebatic acids. Urine octanoyl carnitine and phenylpropionylglycine were not elevated. The child is now 3 y of age, and is doing well without treatment.

Line 1863 was from a male infant with no apparent congenital anomalies who was diagnosed as having GA2 at 2 mo of age on the basis of urine organic acid analysis. He died at home during the 1st y of life, and autopsy was not performed.

Line 1865 was from a 19-wk female abortus in whom the diagnosis of GA2 was made by detection of increased glutaric acid in amniotic fluid and whose kidneys were enlarged secondary to renal cystic dysplasia. The first child of these parents, a girl, had multiple craniofacial anomalies including low set ears, flat facies, and occipital prominence, and had died at 2 d of age with metabolic acidosis, "the smell of sweaty feet," and organic acids typical of GA2. Autopsy had shown large cystic kidneys filling the abdomen, and cardiomegaly with ventricular hypertrophy.

Line 1879 was from a male infant with mild facial dysmorphism who developed transient hypoglycemia, acidosis, hypotonia, and possible airway obstruction during the first 11 d of life. Ethylmalonic and adipic acids were slightly elevated in urine. He did not improve on riboflavin, carnitine, and a low-fat diet, and died at 3 mo of age. Autopsy showed only possible immune glomerulopathy without renal cystic dysplasia, and diffuse gliosis most marked in the brainstem.

Line 1890 was from a North American Indian male infant who presented with hypotonia, acidosis, and hypoglycemia, and died at 19 d of age. Urine organic acids were diagnostic of GA2, and autopsy showed diffuse fatty infiltration of the heart and skeletal muscles, microvascular fat in hepatic parenchymal cells, and small kidneys with a few cysts and dysplastic changes. Fibroblasts from this patient were previously reported to be in the same complementation group as others deficient in ETF-QO (19).

Line 1894 was from a female infant without obvious congenital anomalies, who was diagnosed as having GA2 on the basis of urine organic acid analysis. The child, who was apparently doing well on carnitine and a diet low in protein and fat, died at home, and autopsy was not performed.

Line 1903 was from a baby with an unexplained episode of metabolic acidosis during the newborn period, and who later developed feeding difficulty and became irritable. At 8 mo she had proximal muscle weakness and profound hypotonia, and then deteriorated further, intermittently assuming dystonic postures. Organic acid analysis showed small amounts of compounds typical of GA2. She died at 4 y of age and after autopsy showed dilatation of the lateral ventricles and symmetrical shrinkage of the caudate and putamen with normal globus pallidus. The activity of glutaryl-CoA dehydrogenase in fibroblasts was assayed and found to be normal. Lines 1904 and 1905 were from the mother and father of the child, who were first cousins.

Line 1916 was from a female infant who presented during the 1st d of life with hypoglycemia, hypothermia, metabolic acidosis and a "smell of sweaty feet"; she developed hyperammonemia shortly thereafter, and died on the 11th d of life. Autopsy showed

enlarged kidneys containing numerous fluid-filled cysts, and marked accumulation of lipid in hepatocytes and cardiac myocytes.

Line 1918 was from a female infant with a high forehead and several other dysmorphic features, who developed acidosis, hypoglycemia, and an acid odor during the 1st d of life, with urine showing the typical organic acid pattern of GA2. At 7 d of age, when the diagnosis was made, serum carnitine values were: total carnitine, 39.2 μM , free carnitine, 2.2 μM , and esterified carnitine, 37 μM (normal mean values: total carnitine, 50 μM , free carnitine, 45 μM , and esterified carnitine, 5 μM). She was treated with oral carnitine and riboflavin. After 11 d of carnitine and riboflavin therapy, serum carnitine values were 57.8 μM total, 26.2 μM free, and 31.6 μM esterified. An echocardiogram at 14 days of age showed a severe cardiomyopathy, but a repeat study was normal 2 mo later. She remained hypotonic and developmentally delayed, and died suddenly at 13 mo of age with acidosis and hypoglycemia. Autopsy showed marked fatty degeneration of the heart, and moderate infiltration of the liver.

Line 1935 was from a 19-y-old woman who had developed progressive weakness and intolerance to fasting, with shaking and sweats relieved by oral sweets during childhood. She was hospitalized when she was 18 y old with marked fatigue, muscle weakness, and hepatomegaly, which cleared spontaneously. Three mo before death, an episode of marked muscle weakness and hypoglycemia responded to glucose, and at this time she showed proximal upper and lower limb myopathy, hepatomegaly, and mild hyperammonemia, and a skeletal muscle biopsy demonstrated a lipid storage myopathy. Urine organic acids were typical of GA2 and were normalized with intravenous riboflavin and carnitine. She died from adult respiratory distress syndrome secondary to aspiration. Autopsy showed a fatty liver and heavy, consolidated lungs.

Line 1966 was from a girl whose 1st y of life was normal except for episodes of vomiting, lethargy, and pallor at 5 and 6 mo of age. The first episode responded dramatically to i.v. glucose, but the second progressed to respiratory arrest from which the patient recovered. Then 3 mo later, when she was well, urine organic acid analysis showed large amounts of adipic, moderate amounts of sebatic and suberic acids, and small amounts of glutaric acid. Of three urines obtained an additional 3 mo later, when she was again clinically well, one showed moderate dicarboxylic aciduria with dodecanedioic acid but without glutaric acid, one showed minimal dicarboxylic aciduria with dodecanedioic acid with glutaric acid, and another was entirely normal.

Enzyme preparations and assays. MCAD was prepared as described (20) and had a $A_{270\text{nm}}/A_{448\text{nm}} = 5.3\text{--}5.6$; its concentration was determined using $\epsilon_{448\text{nm}} = 15.4 \text{ mM}^{-1}$ (21). ETF was purified essentially as described by Husain and Steenkamp (22) and had a $A_{270\text{nm}}/A_{436\text{nm}} = 5.8\text{--}5.6$; its concentration was determined using $\epsilon_{436\text{nm}} = 13.4 \text{ mM}^{-1}$ (2). ETF-QO was purified essentially as described by Beckmann and Frerman (7) and its concentration was determined using $\epsilon_{430\text{nm}} = 24 \text{ mM}^{-1}$.

Fibroblasts from two 490 cm^2 roller bottles were trypsinized, washed twice with PBS, and the pellets were stored at -70°C ; ETF and ETF-QO activities were stable in the frozen fibroblasts for at least 3 mo. Pellets were thawed and suspended in 0.5 mL of 10 mM HEPES(K^+), pH 7.8, containing 10% ethylene glycol immediately before enzyme assay, disrupted by sonication, and centrifuged for 1 h at $100\,000 \times g$. Supernatant fractions were assayed for ETF activity and membrane fractions were detergent extracted and assayed for ETF-QO activity (3).

ETF was assayed at 25°C in 0.6 mL reaction mixtures containing 20 mM Tris-HCl, pH 7.5, 130 μM octanoyl-CoA, 70 μM Q_1 , 0.4 μM MCAD (as flavin), and 0.25 μM ETF-QO; reactions were initiated by the addition of 30–150 μg of soluble fibroblast protein, and the reduction of Q_1 was assayed spectrophotometrically at 275 nm ($\epsilon_{275\text{nm}} = 7.3 \text{ mM}^{-1}$) (23). Saturating concentrations of MCAD, octanoyl-CoA, ETF-QO, and Q_1 were deter-

mined in preliminary experiments. Nonspecific decrease in absorbance at 275 nm was determined for each sample in reaction mixtures lacking ETF-QO. Figure 1 shows the dependence of the rate of Q_1 reduction upon added soluble fibroblast protein and the ETF-QO-independent rates that were subtracted from the ETF-QO-dependent rates. Variation of activity using the same extract was less than 10% and variation of activity between different extracts from the same fibroblast line assayed at different times was less than 15%. ETF activity was stable for at least 2 d when the soluble supernatant fractions were stored frozen at -70°C . This assay is capable of detecting at least 0.5 nM ETF when calibrated with purified porcine ETF and is six to eight times more sensitive than a previously described dye-coupled assay and does not require the isolation of a fibroblast mitochondrial fraction (5). ETF-QO was assayed by comproportionation of 2-electron reduced ETF and oxidized ETF (3, 7). Protein was determined by the method of Lowry *et al.* (24) as modified by Miller (25). In all cases, 1 mU of enzyme activity is equal to 1 nmol of product formed per min at 25°C .

Antibody preparations. The IgG fractions obtained by $(\text{NH}_4)_2\text{SO}_4$ fractionation of rabbit antisera against porcine ETF and ETF-QO (3) were affinity purified on 1-mL columns of ETF-QO coupled to Affigel-15, or ETF coupled to Affigel-10. The IgG fractions were dialyzed against 10 mM Tris-HCl, pH 7.4, and passed through the column, equilibrated with the same buffer, three times. The column was washed until no protein eluted, and the specific antibodies were eluted with 0.1 M glycine-HCl, pH 2.7. Antibody-containing fraction were immediately neutralized with 1 M Trizma base, concentrated, dialyzed against 10 mM Tris-HCl, pH 7.4, and stored at 4°C in the presence of 0.04% sodium azide at a concentration of 0.5–1.0 mg/mL.

Electrophoresis and immunoblotting. Fibroblast pellets stored at -70°C were suspended in 10 mM Tris-HCl, pH 7.4, containing 1 mM each of phenylmethyl sulfonyl fluoride, tosyl-L-lysine chloromethyl ketone, and tosylamide-2-phenylethyl chloromethyl ketone and sonicated and centrifuged as described above. Soluble and particulate proteins (80 μg) were electrophoresed on 10–15% polyacrylamide gels in the presence of SDS (26) and transferred to nitrocellulose (27). ETF and ETF-QO were detected with the appropriate antibody and alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin or ^{125}I -protein A.

Materials. The water-soluble ubiquinone homolog, Q_1 , was the generous gift of Eisai Chemical Corp., Kyoto, Japan. ^{125}I -labeled protein A was purchased from ICN Radiochemicals, Costa Mesa, CA. Alkaline phosphatase-conjugated goat anti-rabbit Ig was from Promega Biotec, Madison, WI. Affigel-10 and Affigel-15 were obtained from Bio-Rad Laboratories, Richmond,

CA. Octanoyl-CoA was from Sigma Chemical Co., St. Louis, MO. All other chemicals were the best grade commercially available.

RESULTS

ETF and ETF-QO in GA2 fibroblasts. Table 1 shows the electron transferase activities of ETF and ETF-QO in extracts of control and GA2 fibroblasts. Nine patients with neonatal onset GA2 were deficient in ETF activity and eight were deficient in ETF-QO activity. Of six patients with late onset GA2, three were deficient in ETF, and three were deficient in ETF-QO.

ETF-QO activity in fibroblasts from the parents of patient 1846, and ETF activity from the parents of patient 1903, were intermediate between patient and control values. These observations confirm earlier indications that deficiencies of ETF and ETF-QO are inherited as autosomal recessive traits (3).

Figures 2 and 3 show the results of immunoblotting experiments that were used to determine whether there were major changes in the steady state levels or mol wt of ETF subunits or ETF-QO. Entire gels are shown in Figures 2B and 3 A and B. Immunoblots of the two proteins in some of these lines, *i.e.* 1441, 1196, 1730, and 1691, have been presented previously (3) and are presented here for sake of completeness with the development of the catalytic assay for ETF. Among the ETF-deficient

Table 1. *ETF and ETF-QO activity in GA2 fibroblasts*

	ETF mU/mg protein	ETF-QO mU/mg protein
Control range (mean \pm SD)	1.10–2.50 1.70 \pm 0.40; <i>n</i> = 11	5.4–20.5 14.1 \pm 3.9; <i>n</i> = 20
ETF deficiency		
Neonatal onset		
1441	0.20	12.7
1196	0.05	13.5
1728	0.32	9.8
1902	\leq 0.01	13.0
1803	0.11	5.5
1863	\leq 0.01	11.3
1894	0.08	9.1
1916	0.05	4.4
1918	0.15	9.3
Late onset		
1851	0.48	15.3
1903	0.10	18.6
1966	0.03	10.7
ETF-QO deficiency		
Neonatal Onset		
1730	3.8	\leq 0.1
1691	2.7	\leq 0.1
1692	1.8	\leq 0.1
1808	2.3	0.4
1846	1.8	\leq 0.1
1865	2.6	\leq 0.1
1879	1.5	\leq 0.1
1890	1.3	\leq 0.1
Late Onset		
1591	0.7	2.5
1592	1.2	2.9
1935	1.8	2.9
Parental lines		
1847*	1.10	4.3
1848*	Not done	4.8
1904†	1.00	Not done
1905†	1.00	Not done

* Parents of 1846.

† Parents of 1903.

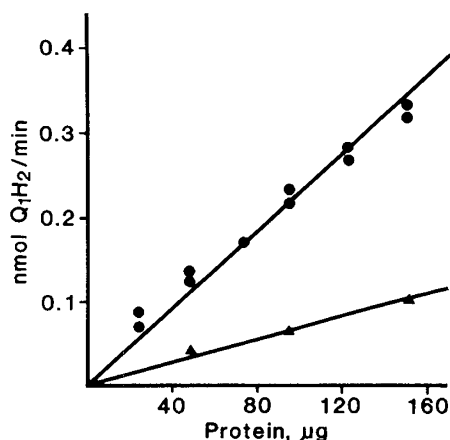


Fig. 1. Assay of ETF in a crude extract of human fibroblasts; dependence of Q_1 reduction upon the concentration of added fibroblast protein. ETF activity was assayed as described in Materials and Methods. The ETF-QO dependent (●) and ETF-QO independent (▲) reduction of Q_1 are indicated.

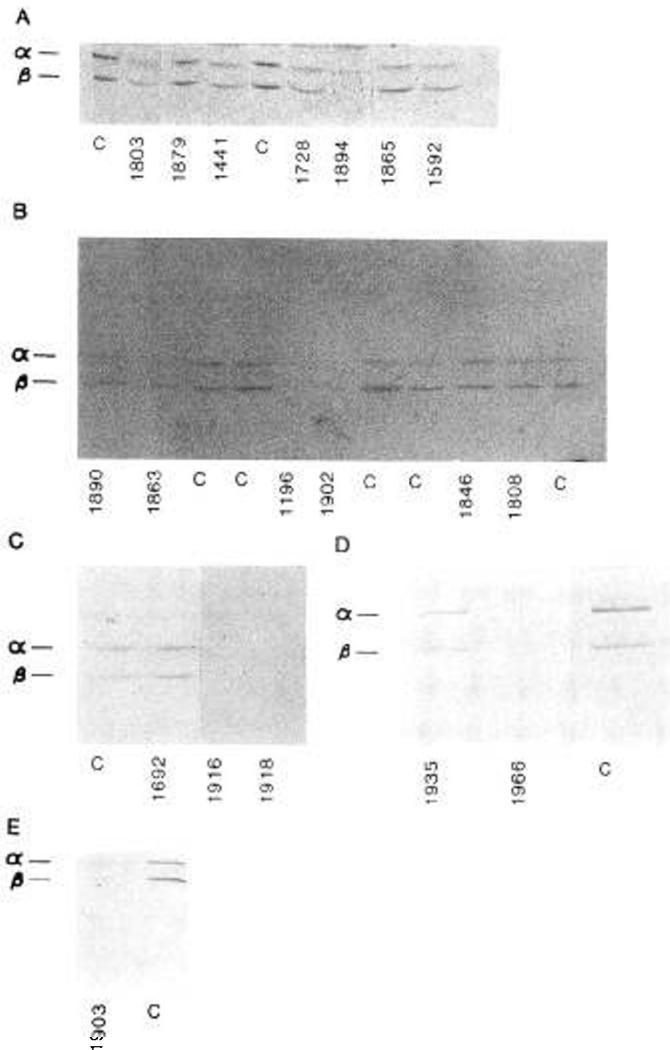


Fig. 2. Immunoblots of fibroblast extracts for α - and β -subunits of ETF. Control lines are in lanes designated C.

lines of particular interest, line 1894 appears to be deficient in the smaller β -subunit and line 1863, which has no detectable ETF activity, contains both subunits with apparently normal mol wt. Line 1916, derived from a patient with renal cysts and deficient in ETF activity contains no detectable ETF antigens. Further, in our previous study (3), patients without detectable ETF-QO activity all lacked detectable ETF-QO antigen. In this investigation, line 1879 has no detectable ETF-QO activity but contains ETF-QO antigen of normal mol wt and at a level comparable with control lines. The lower band in line 1935 (Fig. 3C) may reflect a truncated form of the protein.

DISCUSSION

Detailed biochemical investigation of a larger population of GA2 patients reveals that the clinical phenotype of GA2 is caused as often by a defect in ETF as by a defect in ETF-QO. In ETF deficiency, the residual electron transferase activity varies considerably and, in some cases without relation to clinical severity. For example, patient 1966, with a mild clinical course and intermittent organic aciduria, had lower ETF activity than several patients whose disease presented in infancy. This observation may reflect the relative instability in crude extracts of residual ETF activity in the different mutants. In contrast, the loss of ETF-QO activity was usually complete and antigen was usually undetectable in all patients with ETF-QO deficiency except patients 1808 and 1935, who had milder disease, and most of these patients had renal cystic dysplasia.

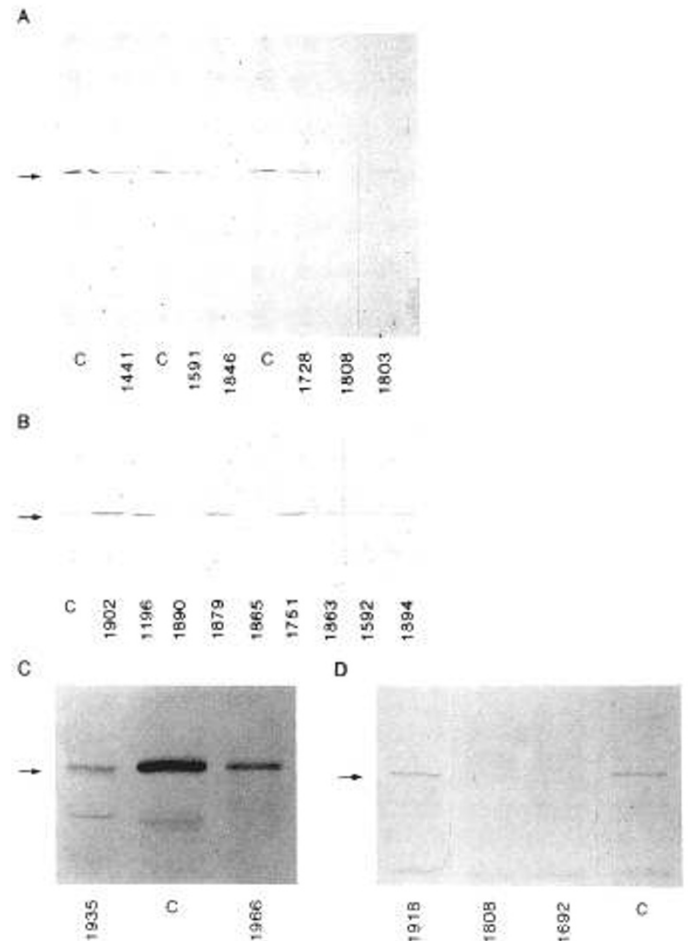


Fig. 3. Immunoblots of fibroblast extracts for ETF-QO antigen. Control lines are in lanes designated C.

The results obtained with patient 1591 are not clear. The patient's fibroblasts had 17% of mean ETF-QO activity of controls when assayed by comproportionation of ETF_{ox} and $\text{ETF}_{2\text{e-}}$, and 23% of mean activity of controls when assayed in the NADH-ETF reductase reaction (12) (data not shown). ETF activity was 42% of the mean activity of controls. Unfortunately no internal control of mitochondrial enzyme activity, *e.g.* citrate synthase, was assayed that might help clarify this ambiguity.

The results on patient 1916 show that renal cystic dysplasia can also occur in ETF deficiency, suggesting that the malformations in these patients is due to either the decreased rate of electron transport or, if the genes for ETF and ETF-QO are closely linked, to a deletion that involves another gene as well. It is not clear why the malformations are more common in ETF-QO deficiency, unless deficiency of ETF-QO is more complete than that of ETF. The fact that ETF-QO antigen is often absent in ETF-QO deficiency supports this contention, but anomalies are often absent in patients without detectable ETF antigen. The striking similarity of the anomalies in GA2 to those of Zellweger syndrome, almost certainly a disease of peroxisomes and not mitochondria, suggests a common pathogenic mechanism. ETF and ETF-QO were normal in the only Zellweger cell line that we have examined.

Patient 1903, with ETF deficiency, is the first GA2 patient reported with extrapyramidal movement disorder, and neuronal degeneration and gliosis in the basal ganglia. These findings are more typical of glutaryl-CoA dehydrogenase deficiency (glutaric acidemia type I), and suggest that glutaric acid, the only compound that is accumulated in both diseases, is the cause of the striatal degeneration. Several suggestions have been made for how such toxicity could occur, including inhibition of neuronal

glutamate decarboxylase or synaptosomal high affinity uptake of glutamate, and accumulation of quinolinic acid (28).

It is important to note that patients 1851 and 1966, both with mild clinical disease, had barely detectable and intermittent organic aciduria, a feature that may make diagnosis difficult unless the abnormalities are present at the time of the initial examination.

The absence of detectable ETF and ETF-QO antigens does not necessarily mean that the polypeptide is not synthesized; rather, the absence of the antigen on immunoblots simply reflects the steady state level of the antigen compared with the level in a control fibroblast line. For example, our experiments did not detect ETF subunits in cell lines 1196 and 1902, whereas pulse labeling experiments show that both lines synthesize the smaller subunit (4). Immunoprecipitable precursor of the larger ETF subunit was not detected in line 1196, and was significantly decreased in line 1902, although the smaller subunit was pulse labeled in both cell lines. The difference between the two approaches lies in the fact that the immunoblotting technique does not differentiate between the capacity of the cell to synthesize the polypeptide and the stability of the peptide if it is synthesized. However, pulse labeling can indicate that the polypeptide is synthesized, but does not indicate whether the polypeptide is metabolically stable unless appropriate chase experiments are carried out. Also, in the case of cell line 1808, which contains about 3% residual ETF-QO activity, we could not demonstrate ETF-QO antigen. This observation may reflect the sensitivity of the immunoblot method, instability of the antigen, or a mutation that affects a dominant epitope.

The assay for ETF we described was developed to overcome the possibility that assays using artificial electron acceptors might not be sensitive to mutations affecting a possibly distinct ETF-QO binding site on ETF. This assay uses only physiologic ETF reductant and oxidant, *i.e.* MCAD and ETF-QO, and was adapted from a published assay for ETF-QO (5). The results show that the assay can be used to characterize GA2 cell lines, and that it is specific and does not require the isolation of fibroblast mitochondria for the sensitive detection of ETF activity.

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