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Riboflavin-responsive complex I deficiency

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Abstract

Three patients from a large consanguineous family, and one unrelated patient had exercise intolerance since early childhood and improved by supplementation with a high dosage of riboflavin. This was confirmed by higher endurance power in exercise testing. Riboflavin had been given because complex I, which contains riboflavin in FMN, one of its prosthetic groups, had a very low activity in muscle. Histochemistry showed an increase of subsarcolemmal mitochondria. The low complex I activity contrasted with an increase of the activities of succinate dehydrogenase, succinate-cytochrome *c* oxidoreductase and cytochrome *c* oxidase. Isolated mitochondria from these muscle specimens proved deficient in oxidizing pyruvate plus malate and other NAD^+-linked substrates, but oxidized succinate and ascorbate at equal or higher levels than controls. Two years later a second biopsy was taken in one of the patients, and the activity of complex I had increased from 16% to 47% of the average activity in controls. In the four biopsies, cytochrome *c* oxidase activity correlated negatively with age. We suspect that this is due to reactive oxygen species generated by the proliferating mitochondria and peroxidizing unsaturated fatty acids of cardiolipin. Three of the four patients had low blood carnitine, and all were found to have hypocarnitinemic family members.

Keywords: Carnitine; Complex I deficiency; Encephalomyopathy; Lactic acidemia; Mitochondrion; Myopathy; Oxidative phosphorylation; Riboflavin

1. Introduction

The large number of reports about mitochondrial DNA mutations, contrasts with the modest number of those about therapy of defects in oxidative phosphorylation [1–30]. This is surprising, because there are many good reasons to study the therapy of these patients. The number of patients with mitochondrial problems is steadily increasing, and effective treatment should be a constant concern to scientists. The insight into the pathophysiology of the diseases involving oxidative phosphorylation is not at all in pace with the knowledge of the molecular biology. The best proof of understanding the mechanisms of their pathophysiology is to alleviate the condition. The reasons why treatment of the patients may be successful are many fold, such as the existence of vitamin-responsive inborn errors of metabolism, the high residual activity often encountered in these mitochondrial defects, the plasticity of the mitochondria, the fact that a small increase in activity of a deficient enzyme may have a high effect on the metabolic flux, the availability of many safe drugs, carnitine (recently reviewed in Ref. [6]) and vitamins, the efficacy of which can be tested by exercise tests, and finally the fact that searching a cure is important for patients (family), clinicians and their relationship.

It is true that therapy in defects of oxidative phosphorylation is often disappointing, but an increasing amount of reports on single patients, indicates that therapeutic attempts could be successful. Table 1 summarizes reports of successful therapy in primary oxidative phosphorylation defects. Criticists could argue that these 'anecdotical' reports prove nothing, since the natural history of these diseases is not known well enough and the improvement cannot be excluded to be part of the course of the disease. Another view is that therapeutic successes are more readily published than failures, and less frequently as suggested by literature [3].

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It is the purpose of this paper to report the investigations in four patients with riboflavin-responsive complex I deficiency.

2. Materials and methods

2.1. Clinical findings

Two girls CV (11 years), CB (15 years), a man JJ (22 years) and his sister MJ (24 years) suffered from easy fatiguability and exercise intolerance since early childhood. On routine examination no muscle weakness was found. They had lactic acidemia of 7.8, 6.5, 2.7 and 3.0 mmol/l (controls up to 1.9 mmol/l). Serum creatine kinase was slightly elevated in the man (177 U/l, controls up to 110 U/l), and normal in the others. Cardiac, pulmonary, visual and renal dysfunction were excluded in all patients.

CV complained of pain in her legs after walking 100–200 m, and had dyspnoea and palpitations on exercise.

CB was completely exhausted and nauseated after 5 km of cycling, the distance to school. She failed to cycle this distance with adverse wind. She had one stroke-like episode at the age of 13 years, with left-sided weakness. She was short for her age.

JJ had to cycle 15 km to school, and had experienced the same problems as CB. After exercising, he experienced pain at the level of the stomach. At the age of 23 years, he had a brief episode of blurred vision, and a headache which disappeared after 2 days. He had no migraines. His work required strenuous exercise, but he had less endurance power than his colleagues.

MJ had the same problems as her brother, and often went to school (15 km) by bus. She had not experienced a stroke-like episode. She gave birth to a son, and during the pregnancy she felt very tired from at the beginning of each day.

The other family members could exercise normally and had no problems with cycling, including the mother of JJ and MJ. Her plasma lactate was 0.3 mmol/l.

An open muscle biopsy was taken from CB, CV and JJ, respectively. After the finding of a complex I deficiency in biopsied muscle a therapeutic trial was started. Per day, and per os, CV received a mixture of vitamins (biotin 20 mg, riboflavin 100 mg, nicotinamide 200 mg), amino acids for the production of Krebs cycle intermediates (L-valine 1 g, L-isoleucine 1 g, L-aspartate 1 g) and DL-carnitine-HCl (2 g). CB received 10–100 mg riboflavin, JJ and MJ 100 mg riboflavin. Directly after therapy increased endurance power was reported by the patients and exercise related nausea and pains disappeared. CB had no more stroke-like episodes. After 1–2 years of riboflavin therapy, their resting lactate decreased and the exercise tolerance assessed by ergometry increased. Later on, no further improvement was observed. CV stopped therapy and reinstitution of

Table 1
Successful therapy in defects of oxidative phosphorylation

Defect(s)	Therapy	1st author, year, Ref.
Kearns-Sayre	Thiamine	Lou, 1981 [7]
Complex I	Riboflavin	Arts, 1983 [8]
		Scholte, 1987 [9]
		Griebel, 1990 [10]
		Bernsen, 1991 [11]
		Bernsen, 1993 [12]
		Bakker, 1994 [13]
Complex III	Menadione + ascorbate	Argov, 1986 [14]
Kearns-Sayre	CoQ	Ogasahara, 1985 [15]
		Ogasahara, 1986 [16]
Cytochrome c_1	Ascorbate	Przyrembel, 1987 [3]
MELAS	CoQ	Goda, 1987 [17]
		Yamamoto, 1987 [18]
		Ihara, 1989 [19]
Complex I	Succinate	Kobayashi, 1987 [20]
Complex I with low K_m for NADH	Nicotinamide	Scholte, 1987 [9]
Mitochondrial myopathies	CoQ	Bresolin, 1988 [21]
		Bresolin, 1990 [22]
Complex IV	CoQ	Nishikawa, 1989 [23]
Complex I	Menadione + succinate	Wijburg, 1989 [24]
Kearns-Sayre & pyruvate dehydrogenase	Thiamin + riboflavin	Scholte, 1991 [25]
Complex I and IV	CoQ	Bendahan, 1992 [26]
Adenine nucleotide translocator	Vitamin E	Bakker, 1993bc [27,28]

Zierz and coworkers [29] showed that CoQ supplementation did not raise the CoQ concentration in muscle. The key question is if CoQ supplementation increases its level in the respiratory chain. Ernster and coworkers showed that this is not the case in rats (this volume). Folkerts and coworkers claimed that it is the case in humans (this volume). Matthews et al. [30] demonstrated in 16 patients with a variety of mitochondrial diseases that CoQ, menadione, ascorbate, thiamine and niacin were ineffective in treatment. See also Martens et al. in this volume.

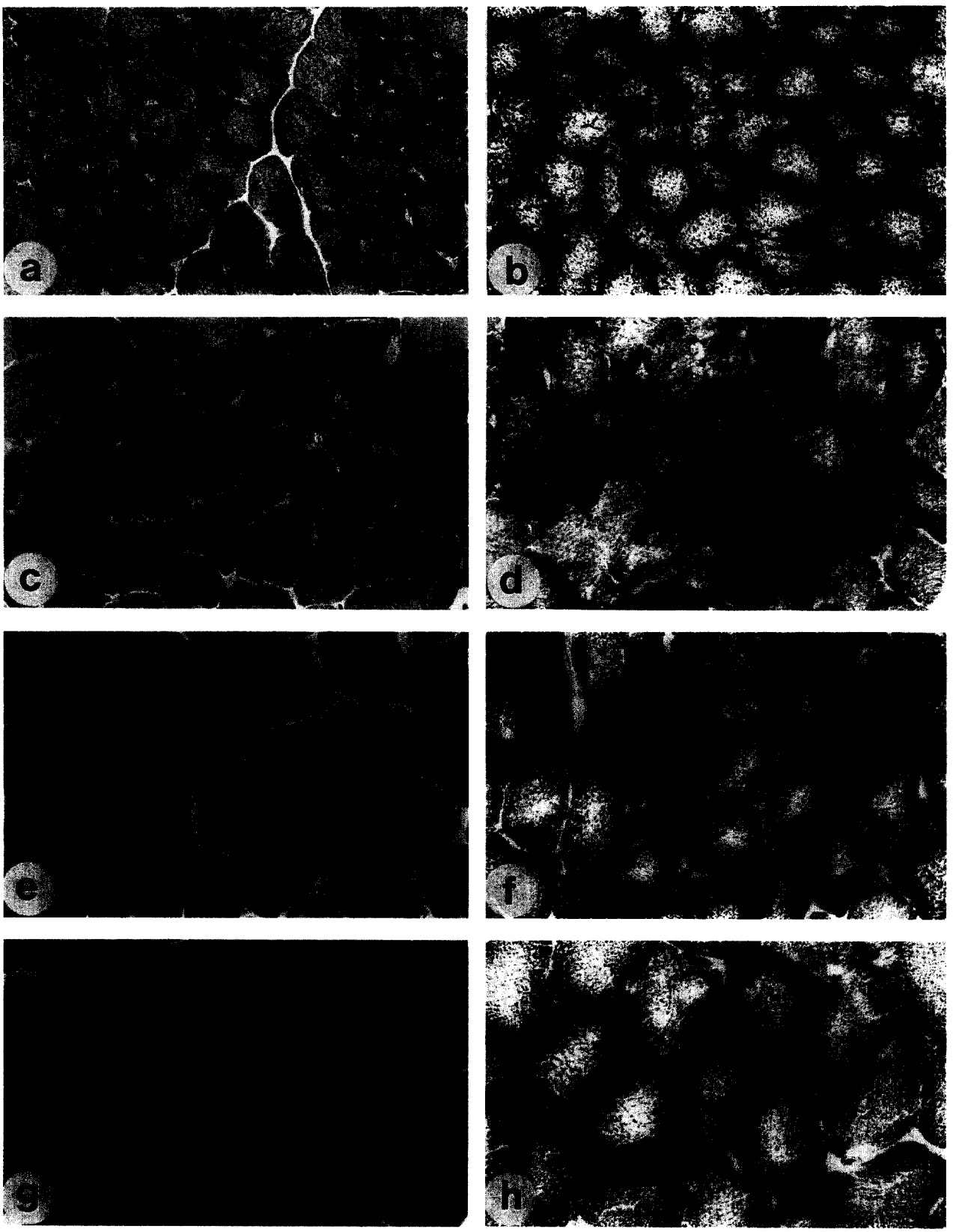

Fig. 1. Left Gomori trichrome staining, right succinate-tetrazolium oxidoreductase. Patient CV: 1a, 1b. Patient CB: 1c, 1d. Patient JJ_1 (first biopsy): 1e, 1f. Patient JJ_2 (second biopsy): 1g, 1h.

daily riboflavin (300 mg) plus L-carnitine (2 g) decreased resting plasma lactate and increased the creatine phosphate resynthesis rate after exercise, assessed in the living muscle by ^{31}P magnetic resonance spectroscopy [13].

The patients were diagnosed in 1985 and 1986 and treated since then. In 1992, blood was taken from the sibling and parents of CB and JJ for future investigation of nuclear DNA in Nijmegen, and for the determination of carnitine. The family members recalled the favourable therapeutic changes to the riboflavin therapy in the patients.

Preliminary biochemical results of patient CB were presented in [9], and a report about patient CV appeared recently [13].

2.2. Muscle biopsy, morphological and mitochondrial DNA investigations

CV, CB and JJ were biopsied by the open procedure under local analgesia from the M. quadriceps. Part of the muscle was immediately immersed in ice-cold medium for the isolation of mitochondria. The remainder was snap frozen for later routine histology and histochemistry, homogenate biochemistry and screening of mitochondrial DNA mutations. A piece of muscle was taken out by a special forceps and fixed in glutaraldehyde for electron microscopy. JJ was again biopsied 2 years later, after therapy with riboflavin from the M. extensor *carpi radialis*.

Dr. B.A. van Oost and coworkers screened the muscle samples for deletions and the 'common' MERRF and MELAS mutations, but these were not found.

2.3. Isolation of and assays on muscle mitochondria

Muscle mitochondria were isolated according to Bookelman et al. [31]. The assays were performed as in earlier work [9,32]. For the calculation of oxygen uptake, which was expressed in nmol O_2 per min, HRS learned during a workshop in Innsbruck from Dr. E. Gnaiger and Dr. W.S. Kunz, that in previous work we had overestimated the amount of oxygen in the oxymetric vessel. We therefore recalculated oxygen uptake rates and P/O ratios assuming that the concentration of O_2 in our ions and albumin containing medium at 25° C was 230 μM [33,34] instead of 267 μM.

2.4. Preparation of and assays on muscle homogenate

This was performed as described before [9,32]. Cytochrome *c* oxidase was assayed according to Cooperstein and Lazarov [35], but with a potassium phosphate buffer concentration of 0.1 M Pi, and horse heart cytochrome *c* (Fe^{2+}) without dithionite.

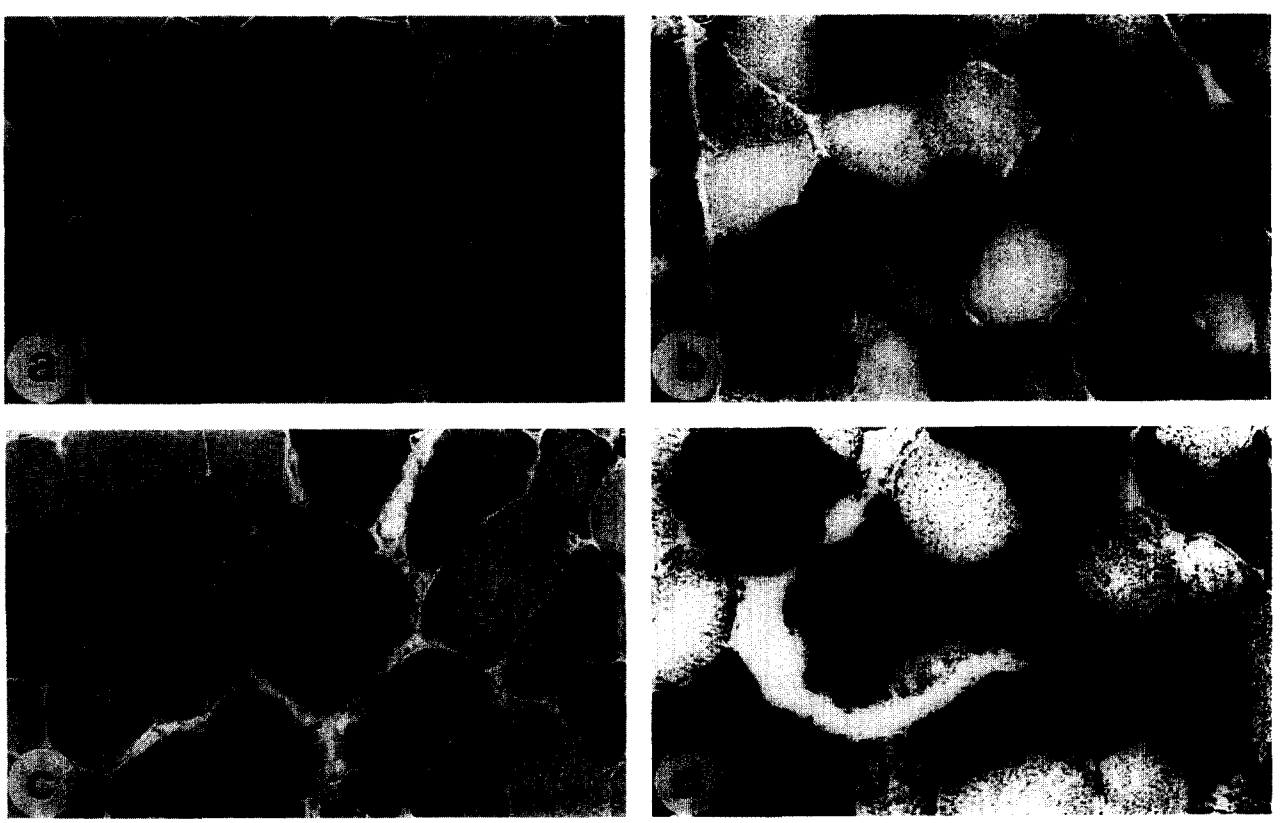

Fig. 2. Stainings see Fig. 1. Patient AD, with complex I deficiency and ragged-red fibres at the age of 15 y: 2a, 2b (patient 1 in Ref. [9]), and at the age of 19 y: 2c, 2d. His mother had the same condition, but a mutation in mitochondrial DNA had not been found (Dr. B.A. van Oost and coworkers).

2.5. Determination of exercise tolerance by bicycle ergometry [36,37]

The exercise protocol was as follows. Metabolic, ventilatory and circulatory variables were measured in the last min of 4 min periods with constant workload, increasing stepwise every 4 min, till the heart rate became higher than 180 beats per min, or till exhaustion. The ergometer-workload was largely independent of pedalling frequency (Hyperbolic type, Lode, Groningen, The Netherlands) and ventilation was measured in the expiratory line with a Lilly type pneumotachometer, which was linear up to 750 $l.min^{-1}$ (Jaeger, Würzburg, Germany). Volume was obtained as integrated flow. Volume calibration was done with a 1 l syringe and correction factors were applied for calculation of the volume at the appropriate conditions of temperature and humidity. The patients breathed via a low resistance two way valve (Jaeger, Würzburg) into a mixing box of 3 l, from which mixed expired gases were sampled. CO_2 was measured with an infrared-analyzer (Jaeger, Würzburg) and O_2 with a paramagnetic analyzer (Taylor Servomex, OA 150, adapted for our purpose by Mijnhardt, Odijk, The Netherlands). The oxygen uptake (V_O) was estimated from the mixed expired O_2 concentration and ventilation (V_e). Lactate was determined at rest and maximal exercise in venous blood from a non-exercising muscle (brachial vein). At 4 occasions, a screening protocol was used in which no ventilatory and metabolic data were obtained, and maximal exercise capacity was determined by the maximal workload, heart rate and lactate levels.

3. Results

3.1. The morphology of muscle and muscle mitochondria

The fibre typing by Ca^{2+}-ATPase, and the distribution and amount of glycogen were as in controls. The amount of fat droplets was slightly increased. The number of subsarcolemmal mitochondria was strongly increased, as shown with Gomori's trichrome stain (Fig. 1, left), and with succinate-tetrazolium oxidoreductase (Fig. 1, right). The sarcoplasm was not fragmented and the fibres had no ragged appearance. The same staining methods were performed in a boy with ragged-red fibres and complex I deficiency at ages 15 and 19 years (Fig. 2). The morphological condition in the patients of Fig. 1 can be described as excessively red, but not ragged. Morgan-Hughes de-

Table 2
Activities of isolated muscle mitochondria [*]

	CV	CB	JJ_1	Controls	n [a]
O_2-uptake rates (nmol O_2)					
Pyruvate + malate + ADP	4	8	10	35(17–50)	22
Glutamate + malate + ADP	5	8	8	35(19–73)	22
Palmitoylcarnitine + malate + ADP	6	9	9	29(17–48)	22
Succinate + rotenone + ADP	50	71	82	45(27–84)	22
Ascorbate + TMPD [b] + ADP	142	301	271	148(99–202)	22
Ascorbate + TMPD + DNP [c]	198	349	277	173(100–224)	9
RCI [d] Pyruvate	0.7	0.9	1.0	2.5(1.5–4.6)	22
RCI Ascorbate	1.4	1.9	2.0	1.7(1.3–2.8)	22
P/O [e] Pyruvate	1.3	2.3	3.4	2.0(1.5–2.3)	5
P/O Ascorbate	0.7	1.2	0.6	0.5(0.3–1.0)	7
Mg^{2+}-ATPase (nmol ATP)	41	27	30	44(0–175)	19
+ uncoupler	1078	885	1135	603(174–1003)	19
Radiopalmitate oxidation (nmol palmitate)					
+ 1 mM KCN + 0.1 mM CoA	0.2	0.5	0.2	0.2(0.1–0.4)	12
+ 0.5 mM L-carnitine + 0.1 mM CoA	1.2	1.4	1.6	1.7(1.1–2.5)	14
+ 5 mM L-carnitine + 1 mM CoA	1.1	1.8	2.7	1.9(1.1–2.3)	9
Malonyl-CoA decarboxylase					
(nmol)	0.1		0.2	0.1(0.0–0.8)	9
+ detergent	2.6		1.9	1.4(0.9–3.1)	9
Succinate dehydrogenase					
(nmol INT^+) [f]	55	54	62	27(12–57)	21
Practical yield					
(mg protein/g wet wt)	17	10	4.6	4.7(1.0–11)	22
Recovery (%)	27	16	14	16(4.9–45)	21

[*] The activities were expressed in indicated amounts of substrate.min^{-1}.(mg protein)$^{-1}$.
[a] n = number of independent controls.
[b] TMPD = tetramethyl-*p*-phenylenediamine used in a concentration of 0.6 mmol/l.
[c] DNP = 2,4-dinitrophenol.
[d] RCI = stimulation of oxygen uptake rate by ADP.
[e] P/O = mol approx. P produced/0.5 mol O_2 consumed.
[f] INT^+ = *p*-iodotetrazolium.

scribed this condition as: 'Rim(med) with red staining granular material, but without a ragged-red appearance' [38]. The staining of type I and also of type II fibres by succinate-tetrazolium oxidoreductase, was more intense than in controls, and the difference between the fibre types was less pronounced than in controls. In patient CV it was no longer possible to distinguish the fibre types in this stain. Cytochrome *c* oxidase staining showed a better difference between the fibre types in patient CV, but the Ca^{2+}-ATPases gave a better contrast. This was also true for patients CB and JJ. There were no cytochrome *c* oxidase negative fibres. A second biopsy from JJ after riboflavin treatment was similar to that before treatment. The fact that the muscle fibres were larger after therapy could be related to the other muscle (quadriceps in Figs. 1e, 1f vs. extensor *carpi* in Figs. 1g, 1h).

Electron microscopy confirmed the subsarcolemmal mitochondrial accumulation. They were abnormal in size distribution. The larger mitochondria showed an abnormal organization of the cristae and an increase in dense bodies. Crystalline inclusions were not found.

3.2. Biochemistry of muscle mitochondria and muscle homogenate

The oxygen uptake rates with the NAD^+-linked substrates were decreased to 11–31% of the average rates in controls (Table 2). This contrasted with the normal oxidation of succinate in the presence of rotenone and the normal (CV) to highly increased oxidation (CB, JJ) of ascorbate plus TMPD. Uncoupler increased this oxidation by 39% (CV), 16% (CB) and 2% (JJ), which is in the range of controls (1–51%). The RCIs with pyruvate plus malate were low (0.7–1.0), which is a common finding with poorly oxidized substrates. The quality of the coupling between oxidation and phosphorylation was demonstrated by the good RCIs with ascorbate plus TMPD. The yields of oxidative phosphorylation, as expressed by the P/O ratios were also good. The integrity of the mitochondria was further demonstrated by the low Mg^{2+}-ATPase activity of the mitochondria, and the impressive stimulation of this activity by uncoupler (26-, 33- and 39-fold, which is much higher than in the average control).

Palmitate oxidation, measured in the presence of cyanide, reflects the peroxisomal β-oxidation. With low (0.5 mM) carnitine, also the mitochondrial contribution was measured, which was in the control range. An explanation for the discrepancy with the low oxidation of palmitoylcarnitine plus malate, is that the latter reaction uses much more oxygen than the former, where acylcarnitine and not CO_2 is an important product [39]. High (5 mM) carnitine did not stimulate the β-oxidation in CV further. In CB there was a clear stimulation, and the greatest stimulation was found in JJ. It is likely that a high stimulation of the mitochondrial β-oxidation by 5 mM carnitine reflects a high intramitochondrial acyl-CoA/CoA ratio [40].

In addition to the uncoupler-stimulation of the Mg^{2+}-ATPase, the high stimulation by detergent of the mitochondrial matrix enzyme malonyl-CoA decarboxylase in patients CV and JJ, indicates the integrity of the mitochondrial inner membranes. Like citrate synthetase, this matrix enzyme reacts with a substrate, which cannot pass the intact inner membrane. The mitochondrial yield in the patients decreased with age (17, 10 and 4.6 mg protein/g muscle). This is not the case in controls. The recovery of the isolation was 14–27%. Isolation of mitochondria always imply the danger of selection. The isolated mitochondria, with their inability of oxidize NAD^+-linked substrates, may not represent the properties of the not isolated mitochondria. For this reason, it is important not only to study isolated mitochondria, but also mitochondrial activities in the homogenate [41]. We investigated a so-called 'total' homogenate, which was not centrifugated, to avoid selection [42]. The results are summarized in Table 3. The activity of complex I, measured as rotenone-sensitive NADH oxidase, was decreased to 9% (CV), 5% (CB) and 16% (JJ_1). After two years of riboflavin therapy the latter activity had increased to 47% (JJ_2), an activity which is in the (lower) range of controls. The activity of succinate

Table 3
Mitochondrial enzymes, creatine kinase, and total carnitine and protein in muscle homogenates

Patients	CV	CB	JJ_1	JJ_2	Controls	*n*
Age (years)	11.9	15.1	22.6	24.7		
Complex I (μmol NADH)	0.33	0.18	0.58	1.71	3.7(1.6–7.3)	45
Succinate dehydrogenase (μmol INT^+)	3.38	3.44	2.00	3.15	0.72(0.19–1.64)	58
Complex II + III (μmol cytochrome c)	11.1	14.8	11.3	13.7	5.2(1.6–12.5)	54
Complex IV (1st order rate constant k)	413	305	257	163	95(45–161)	49
CPT I (nmol L-carnitine)	138	121	119	89	77(43–165)	55
CPT II (nmol L-carnitine)	162	200	122	126	96(57–174)	57
Creatine kinase (μmol creatine)	425	335	388	379	307(83–430)	50
Total carnitine (μmol per g wet wt)	2.49	3.25	2.58	2.57	3.96(2.57–5.73)	59
Protein (mg per g wet wt)	187	175	195	186	174(77–263)	53

The activities are expressed in amount of substrate.min^{-1}.(g wet weight)$^{-1}$.
CPT = carnitine palmitoyltransferase.

dehydrogenase was increased to 278–478% of the average activity in controls, complex II + III to 213–285%, complex IV to 270–435%, CPT I to 155–179%, CPT II to 127–208% and creatine kinase, in contrast to the preceding mitochondrial activities mainly residing in the cytosol, to 109–138%. The last 3 enzyme activities were in, or closer to, the range of controls than the activities of the terminal respiratory chain. Carnitine had about the same level as in the lowest control in patients CV and JJ, and was normal in patient CB.

3.3. Exercise testing

The efficacy of the riboflavin treatment was assessed by bicycle ergometry of patients CB, JJ and MJ (Table 4) and ^{31}P magnetic resonance spectroscopy of patient CV [13]. Before treatment CB could exercise 4 min 30 Watts and was exhausted at a heart rate of 147 beats/min. Her blood lactate before the test was 6.5 mM and increased to 12.8 mM at the end of the test. After receiving 10 mg riboflavin per day she could perform 4 min 30 Watts plus 2 min 60 Watts at a heart rate of 183 bpm, and lactate rose from 3.7 to 10.7 mM. After another year on 100 mg riboflavin her maximal workload had increased to 4 min 30 Watts, 4 min 60 Watts plus 4 min 90 Watts, her heart rate was 186 bpm and lactate rose from 2.5 mM to 14.2 mM. The amount of work performed per amount of oxygen consumed and per amount of lactate produced increased considerably. No more improvement was found in later studies. JJ showed the same, but reached already a heart rate of 180 bpm before treatment. By his daily physical labour he was obviously better trained and could perform more work before therapy than CB. MJ has not been tested before riboflavin treatment. After therapy, two measurements yielded about the same results, but lactate at rest was higher the second time. It is striking that the maximal workloads after therapy were so different in the three patients.

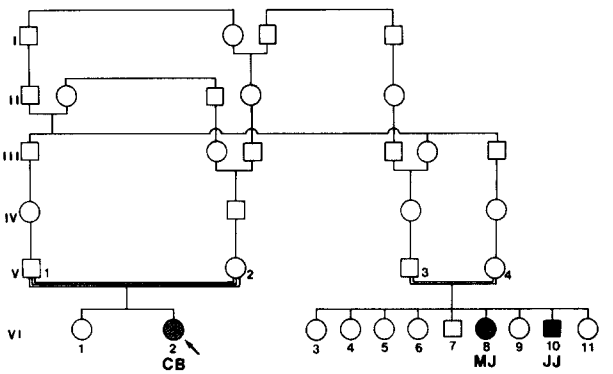

Fig. 3. The pedigree of families B and J. This pedigree was recontructed with the help of the archives of the Genealogical Society of Utah in Salt Lake City, USA.

3.4. Family relationship

Geneological research revealed that CB and JJ were related (Fig. 3). This had been expected because of the great clinical similarity between the patients, and the fact that they lived on the same small Dutch island, which could only be reached by boat until the Delta works were constructed after the Dutch flood disaster of 1953. The implication of this complicated consanguineous pedigree is that the heredity of the condition is autosomal recessive. CV comes from another part of the country, and a relation between her family and that of CB and JJ could not been found. From all available evidence, it is likely that CV has the same condition as CB, JJ and MJ. All other patients we studied with complex I deficiency had a decreased or normal cytochrome *c* oxidase activity, and ragged-red or normal fibres.

3.5. Plasma carnitine

Free and total carnitine in plasma from the families, revealed that the patients CV, JJ and MJ were hypocar-

Table 4
Determination of exercise tolerance by cycle ergometry

Patient	Age	Riboflavin	Workload *		V_E *	V_O *	HR *	LA * *	LA *
	years	mg/day	min	Watts	l/min	nmol/min	/min	mmol/l	
CB	15	0	4	30	37	34	147	6.5	12.8
CB	16	10	2	60	38	31	183	3.7	10.7
CB	17	100	1	90	51	41	186	2.5	14.2
CB	18	25	1	90			183	3.8	15.5
CB	19	25	1	90	50	44	183	3.8	12.8
JJ	22	0	4	60	64	48	180	8.8	14.3
JJ	23	100	1	120	88	76	189	6.2	16.3
JJ	24	100	4	90	71	75	186	4.9	15.5
JJ	26	100	1	120			190	6.9	14.1
MJ	26	100	2	90			198	4.7	14.8
MJ	29	100	2	90			196	8.2	18.3

The workload was stepwise increased from 4 min 30 Watts, 4 min 60 Watts, 4 min 90 Watts etc. till the heart rate became higher than 180 beats per min, or till exhaustion.

* At maximal exercise. * * Before exercise. V_E = ventilation. V_O = oxygen uptake. HR = heart rate. LA = lactic acid.

Table 5
Plasma carnitine in patients and their family members

Person	Pedigree	Carnitine (μmol/l)	
		Free	Total
CV [a]		11	24
CV [b]		30;30	38;45
Father		39	49
Mother [b]		27	36
Brother		21	28
CB	VI,2	34	40
Father	V,1	38	44
Mother	V,2	29	34
Sister	VI,1	24	31
MJ	VI,8	24	31
JJ	VI,10	22;25	31;30
Father	V,3	47	52
Mother	V,4	34	40
Sister	VI,3	22	28
Sister	VI,5	29	32
Sister	VI,6	38	41
Sister	VI,9	28	35
Sister	VI,11	26	36
Controls (40)		40	51
Controls range		26–58	31–74

[a] Just after stopping carnitine supplementation.
[b] Takes oral L-carnitine.

nitinemic. All patients had healthy sib with low blood carnitine (Table 5). The question arises if this genetic background is required to get the mutation in the nuclear-encoded part of complex I.

4. Discussion

The clinical characteristics of these patients with riboflavin-responsive complex I deficiency were the following. The patients suffered from exercise intolerance by fatigue and pain. They had lactic acidosis, showed subsarcolemmal mitochondrial proliferation with increased activities of the terminal respiratory chain. They had neither ragged-red fibres nor succinate-tetrazolium oxidoreductase or cytochrome *c* oxidase negative fibres. They had healthy siblings with low plasma carnitine.

The increase of subsarcolemmal mitochondria in type I and in type II fibres in the patients is greater than that encountered in trained athletes. But the patients were of course not trained at all. This red but not ragged organization of the mitochondria is not pathological, in contrast to that in ragged-red fibres. In the former condition, mitochondria accumulate at a site where they are needed. In the ragged condition they accumulate also in the centre of the fibre and apparently at random, uncontrolled, no longer restrained by the cytoskeleton. It is tempting to speculate that the central localization of the mitochondria makes them more vulnerable to reactive oxygen species, because the proliferation of the mitochondria plus the defect in oxidative phosphorylation give rise to much more of these species than can be destroyed by the cellular protective mechanisms. Moreover these enzymatic and scavenging protection mechanisms may be much lower in the ragged-red fibres and/or less available.

It is of interest that the riboflavin therapy, albeit restoring the complex I activity to the lower normal range, did neither normalize blood lactate nor the proliferation of mitochondria. It is therefore likely that in vivo the activity is still too small. Kinetic measurements of complex I were not performed.

It is fascinating that the activity of cytochrome *c* oxidase in the patients decreases with age. In the four biopsies the correlation coefficient between the enzyme activity and age, was found to be -0.94 (Table 3).

In another wheel-chair bound patient, with lactic acidosis, subsarcolemmal mitochondrial proliferation and deficiency of the adenine nucleotide translocator in muscle, we detected also such a decrease in cytochrome *c* oxidase with age [43]. At the age of 3.5 year his muscle had an activity of 176, expressed in the first order rate constant $k.\mathrm{min}^{-1}.(\mathrm{g\ wet\ weight})^{-1}$, while at the age of 5.5 the activity was 115. In both biopsies, the other mitochondrial enzyme activities were highly increased, and by immunoblotting of the subunits of cytochrome *c* oxidase it became clear that the amount of protein of the subunits had also considerably increased. We attributed this paradoxal decrease in cytochrome *c* oxidase activity to the generation of reactive oxygen species, peroxidizing the cardiolipin required for activity [44] and treated the patient with 1 g vitamin E per day. He improved within a short time, and no longer required his wheel- chair, except for long distances [27]. Since blood lactate did not decline significantly, we concluded that the primary defect had not been cured. We interpreted the improvement following high vitamin E doses as a result of suppression of the generation of reactive oxygen species caused by the mitochondrial proliferation [28]. It will be clear that the patients of this communication are at present also treated with vitamin E, but exercise testing has not yet been performed.

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