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Mitochondrial myopathy involving ubiquinol-cytochrome *c* oxidoreductase (complex III) identified by immunoelectron microscopy

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The distribution of respiratory chain complexes in bovine heart and human muscle mitochondria has been explored by immunoelectron microscopy with antibodies made against bovine heart mitochondrial proteins in conjunction with protein A-colloidal gold (12-nm particles). The antibodies used were made against NADH-coenzyme Q reductase (complex I), ubiquinol cytochrome *c* oxidoreductase (complex III), cytochrome *c* oxidase, core proteins isolated from complex III and the non-heme iron protein of complex III. Labeling of bovine heart tissue with any of these antibodies gave gold particles randomly distributed along the mitochondrial inner membrane. The labeling of muscle tissue from a patient with a mitochondrial myopathy localized by biochemical analysis to complex III was quantitated and compared with the labeling of human control muscle tissue. Complex I and cytochrome *c* oxidase antibodies reacted to the same level in myopathic and normal muscle samples. Antibodies to complex III or its components reacted very poorly to the patient's tissue but strongly to control muscle samples. Immunoelectron microscopy using respiratory chain antibodies appears to be a promising approach to the diagnosis and characterization of mitochondrial myopathies when only limited amounts of tissue are available for study.

Introduction

Mitochondrial diseases involving the components of oxidative phosphorylation are rare and vary in clinical presentation, with different tissues being affected and with variable onset of symptoms (reviewed in Refs. 1 and 2). Diagnosis is generally made on open biopsy samples, initially by the finding of 'ragged red fibers' in light microscopy of samples stained with trichome and by the presence of abnormal mitochondria upon electron microscopic examination of the tissue. More precise diagnosis can now be made by identification of spectral changes and/or alterations in enzymatic activity upon biochemical analysis of the tissue involved. More recently, several cases of mitochondrial myopathy have been examined at the protein level, after isolation of defective mitochondria [3–5].

We have studied several patients with mitochondrial dysfunction (e.g., Refs. 3,4). The best characterized is a female with pronounced lactic acidosis and muscle weakness, shown to result

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from defective complex III in skeletal muscle [3,4]. Complex III in higher eukaryotes is a complex of 11 different polypeptides, cytochrome *b* (M_r 44000), cytochrome c_1 (M_r 31000) and a non-heme iron protein (M_r 28000) being the three major components with prosthetic groups and known function [6,7].

In our complex-III-deficient patient, immunoblotting with antibodies to individual components showed that cytochrome c_1 was present but cytochrome *b*, the non-heme iron protein and core proteins were absent [3,8]. It proved possible to establish a rational therapy for this particular patient; through oral administration of menadione and ascorbate to act as electron donors and acceptors in place of the missing redox components [9].

Diagnosis of mitochondrial diseases by spectral analysis, activity measurements and by immunoblotting requires large amounts of tissue, obtained by open biopsy or at autopsy. Large amounts of tissue are not always available for analysis. Moreover, biochemical analysis depends on disruption of the tissue being studied and it is important to rule out that observed compositional changes in the respiratory chain are not a secondary defect, caused by proteolytic degradation or dissociation and loss of proteins during isolation of mitochondria for characterization. Here we describe examination of tissue from a patient with a mitochondrial myopathy using immunoelectron microscopy. This technique requires only small amounts of tissue, such as are obtained by needle biopsy, and examines the organization of mitochondria in situ, precluding the possibility that respiratory chain alterations resulted from tissue disruption or dispersion.

Materials and Methods

Tissue samples

Control and patient muscle tissue were obtained by open biopsy after signed permission and informed consent were given. The tissue was frozen immediately and stored at −70 °C. Bovine heart tissue was also frozen and stored at −70 °C prior to thin sectioning to provide the appropriate control conditions.

Complex III was prepared from beef heart mitochondria according to the method of Rieske [10] and cytochrome *c* oxidase by the method of Capaldi and Hayashi [11]. Purified complex I was the kind gift of Dr. Y. Hatefi (Scripps Clinic, San Diego, CA). Antibody against complex I was generously provided by Dr. J. Hare (Oregon Health Sciences University, Portland, OR).

Antibody preparation and characterization

Core proteins and the Rieske non-heme iron protein were isolated in denatured form from complex III preparations by gel filtration in Bio-Gel P-100 after dissociated in SDS. Antibodies to the different electron transfer components were raised in rabbits. The specificity of each antibody was tested by ELISA [12] and by immunoblotting. SDS-polyacrylamide gels were made as described by Fuller et al. [13] and Western blots were performed as described by Towbin et al. [14], with the addition of 0.1% SDS to the electrophoresis buffer.

Preparation of protein A-gold probe

Tetrachloroauric acid was reduced by trisodium citrate and tannic acid [15] and the resulting gold colloid conjugated with protein A at pH 6.9. The protein A-gold complex of uniform size (approx. 12 nm diameter) was isolated by centrifugation in a glycerol gradient (10–30%).

Tissue preparation and immunogold labeling

Muscle tissue (bovine heart and human muscle) was thawed and then suspended in 1% glutaraldehyde, 1% paraformaldehyde and 0.12 M sodium phosphate (pH 7.4) for 2 h at 4 °C. The tissue pellet was washed once in the same buffer before reaction with 2% osmium tetroxide for 1 h at 4 °C. The tissue was then dehydrated in graded ethanol and embedded in Lowicryl HM20, with all manipulations done at −20 °C according to the method of Roth et al. [16].

Ultrathin sections cut on an ultramicrotome with a glass knife were mounted on nickel grids for antibody reaction. The thin sectioned grids were rinsed in distilled water for 10 min and then etched in saturated sodium metaperiodate for 30 min. They were reacted against antibody for 1 h at room temperature, washed several times with 0.1% Tween 20/20 mM Tris buffer (pH 8.2) and then reacted with the protein A-colloidal gold complex

for 1 h [17]. After washing with 0.1% Tween 20/20 mM Tris buffer (pH 8.2) several times to remove unbound protein A, the sections were doubly stained with 2% uranyl acetate for 10 min and with lead citrate for 2 min. The labeled specimens were examined using a Philips 300 electron microscope.

Quantitation of labeling with electron transfer protein antibodies

The quantitation of labeling was done with electron micrographs of magnification 30 000 and 50 000. For each antibody, ten different micrographs were evaluated. Masks with holes 1.5 × 0.6 cm and 2.6 × 1.2 cm were used with the lower and higher magnification pictures, respectively. This was moved around each micrograph randomly until ten areas within mitochondria and ten areas within the myofibrils had been found and sampled for the number of gold particles included in each area. Thus, in all, 100 areas in mitochondria and 100 within myofibrils were used for each data point in Table I. Analysis of the myofibrils acted as a control of nonspecific labeling by the different antibody preparations.

Results

Initial experiments to find conditions of sample preparation and thin sectioning that preserved the antigenicity of respiratory chain complexes were carried out with bovine heart tissue. The antibodies used were prepared against bovine heart components, including the intact complex III, the isolated core proteins of complex III, the Rieske non-heme iron protein of complex III, the cytochrome *c* oxidase complex and complex I. The reactivity of each of these antibody preparations has been established by ELISA and by immunoblotting. The results for the first four antibody preparations listed above have been published already [3,4]. The complex I antibody reacted against native complex I and bound to nine of the more than 30 different polypeptides in immunoblots of complex I denatured in SDS. None of the antibodies cross-reacted with respiratory chain complexes other than those to which they were made. Fig. 1 shows the labeling of beef heart mitochondria obtained when ultrathin sections were reacted first with antibody against cytochrome *c* oxidase (A and B) or core proteins (C) followed by protein A-colloidal gold. Conditions were optimized by titering with increasing amounts of antibody, and quantitating the number of gold particles per 0.1 μm^2 in mitochondria (specific) versus myofibrils (nonspecific labeling). There was no labeling of tissue when protein A-gold was added without prior antibody treatment.

The optimal labeling of bovine heart mitochondria by the various antibodies is given in

TABLE I

QUANTITATIVE ANALYSIS OF THE LABELING DENSITY OF THE DIFFERENT RESPIRATORY CHAIN ANTIBODIES IN MITOCHONDRIA (A) AND BACKGROUND (B) OF DIFFERENT TISSUES (GOLD PARTICLES/0.1 μm^2, ±S.D.)

The numbers in parentheses after tissue type indicate the number of different preparations (bovine heart) and number of different tissue samples (human control muscle) used in the analysis.

Tissue	Antibody									
	complex I		core I and II		complex III		non-heme iron		cytochrome *c* oxidase	
	A	B	A	B	A	B	A	B	A	B
Bovine heart (3)	3.44 (±0.11)	0.85 (±0.29)	15.01 (±3.32)	1.85 (±0.54)	26.84 (±3.29)	2.43 (±0.88)	9.74 (±2.26)	0.99 (±0.62)	38.74 (±6.21)	1.12 (±0.39)
Patient muscle	2.59 (±0.90)	0.71 (±0.36)	1.64 (±0.67)	0.74 (±0.34)	1.34 (±0.55)	0.72 (±0.32)	0.97 (±0.53)	0.55 (±0.32)	14.03 (±2.66)	1.57 (±0.64)
Human control muscle (4)	2.01 (±0.46)	0.39 (±0.28)	8.81 (±1.05)	0.92 (±0.26)	10.86 (±0.74)	2.06 (±0.44)	10.87 (±0.54)	0.76 (±0.16)	13.57 (±1.82)	1.46 (±0.65)

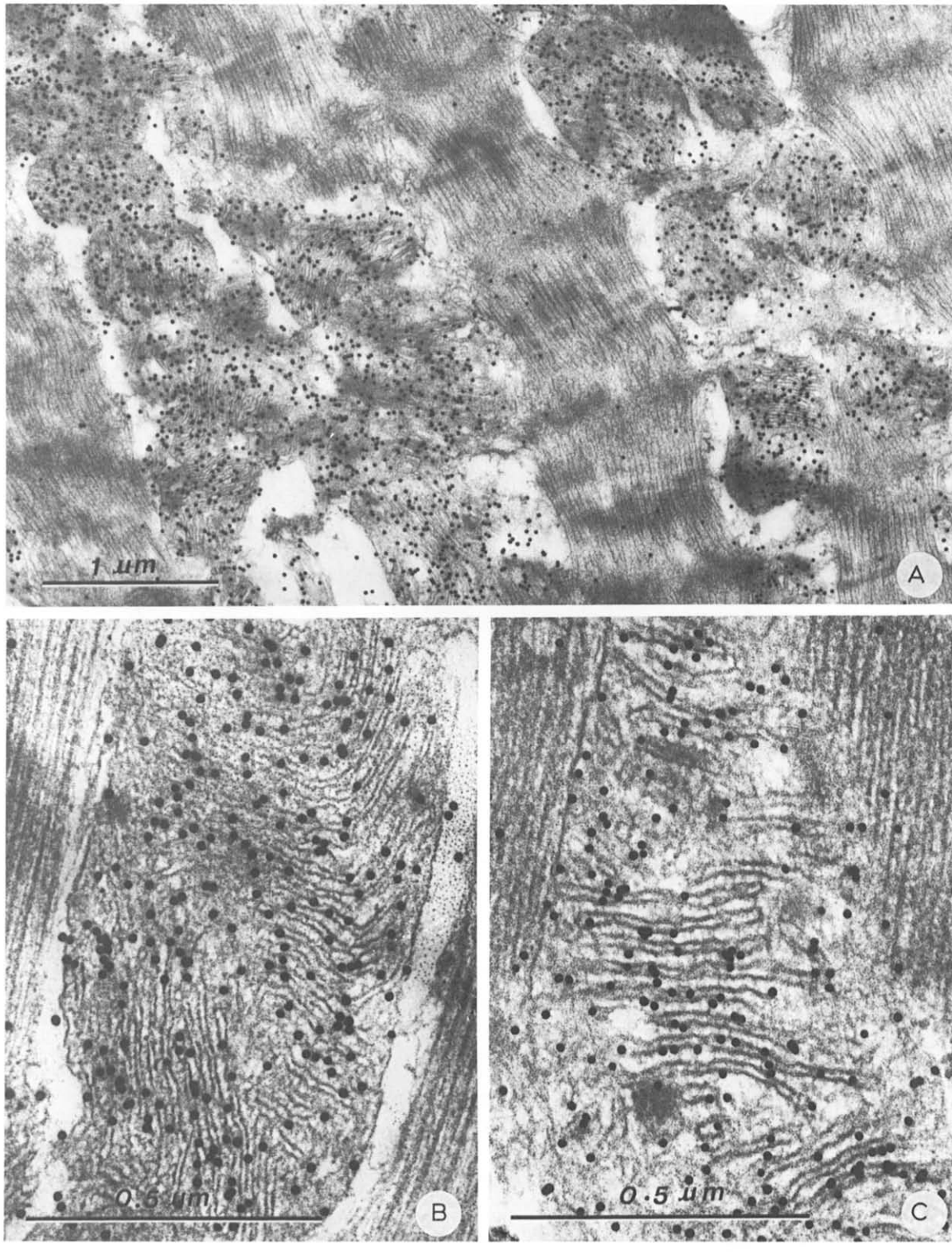

Fig. 1. Electron micrographs of a section of bovine heart tissue, labeled with antibodies against cytochrome *c* oxidase (A and B) and core proteins of complex III (C), followed by protein A-colloidal gold 12-nm particles.

Table I. The ratio of labeling by antibodies to complexes I, core proteins (as a measure of complex III) and cytochrome *c* oxidase of approx. 1 : 5 : 10, respectively, is roughly proportional to the abundance of these respiratory chain components in the mitochondrial inner membranes, as determined by biochemical studies [7]. The labeling by anti-complex III antibodies was higher than that obtained for core protein. It may be that the polyclonal antibodies made against the entire complex III can react more than once with a single complex III molecule, for example, from both sides of the membrane. In contrast, the more specific anti-core protein antibodies and anti-cytochrome *c* oxidase antibodies (reacting primarily with subunits C_{IV} and C_{VI}) may react only once per complex because of steric constraints. High magnification micrographs show the colloidal gold particles attached to the inner membrane and randomly distributed on the membrane whether the labeling was done for complex III (by core protein antibody; FIg. 1(C)) or cytochrome *c* oxidase (Fig. 1(B)).

The conditions of sample preparation and colloidal gold labeling developed for bovine heart tissue proved to work well for human muscle tissue and were used in a comparison of respiratory chain integrity for a patient with a well-

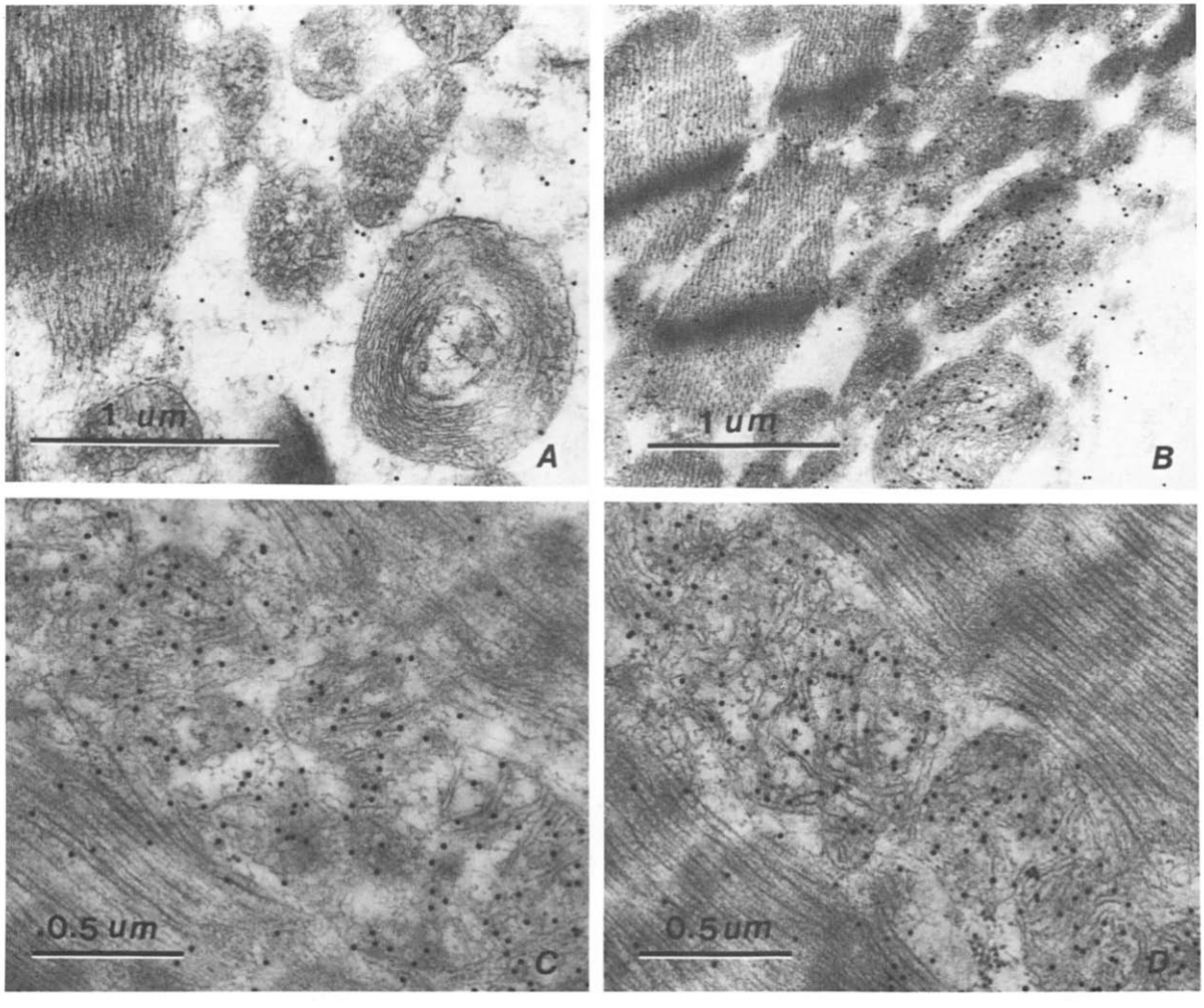

Fig. 2. Electron micrographs of sections of patient muscle (A and B) and human control muscle (C and D) labeled with antibodies against cytochrome *c* oxidase (B and D) and core proteins of complex III (A and C).

defined complex III deficiency and control human tissue. Fig. 2 shows electron micrographs of patient (A and B) and control muscle tissue (C and D) labeled for complex III with core protein antibody (A and C) and for cytochrome *c* oxidase (B and D).

Previous biochemical studies have established that the skeletal muscle of this patient contains less than 10% of complex III activity and less than 10% of cytochrome *b* (as detected spectrally) of normal human muscle samples [3]. Succinate dehydrogenase, cytochrome *c* oxidase and ATPase activities were normal in the patient, localizing the respiratory chain defect to complex III [3]. The electron micrographs in Fig. 2 confirm the strange structure of muscle mitochondria seen in initial pathological examination of the patient tissue. Many of the mitochondria show whorls or concentric rings of membranes. As shown in Fig. 2, these mitochondria label for cytochrome *c* oxidase but not for complex III, whether reacted with antibody to core proteins (as in the figure), nonheme iron protein or whole complex III. A quantitation of this labeling in comparison with normal human tissue is given in Table I. The electron microscopic data show clearly that the patient is defective in complex III and rule out the possibility that this defect is generated during isolation of mitochondria for biochemical analysis.

Discussion

The mitochondrial electron-transport chain is a complicated assembly of four large multipeptide complexes localized to the mitochondrial inner membrane. NADH-ubiquinone reductase (complex I), succinate ubiquinone reductase (complex II), ubiquinone-cytochrome *c* oxidoreductase (complex III) and cytochrome *c* oxidase (complex IV) contain over 30, 5, 11 and 13 different polypeptides, respectively, and respiratory chain activity also requires cytochrome *c* and ubiquinone or coenzyme Q [7]. Most of the polypeptides of the respiratory chain are coded for in the nucleus and made on cytoplasmic ribosomes, but seven subunits of complex I, one subunit of complex III (cytochrome *b*) and three subunits of cytochrome *c* oxidase are coded for on mitochondrial DNA and made inside the mitochondrion [18,19].

Defects of the mitochondrial respiratory chain can occur through mutations in the nuclear coded subunits, by alteration of mtDNA or by mutation of proteins required for the biosynthesis of the mitochondrial gene products.

Mitochondrial myopathies involving complexes I, III and IV individually or in combination have been described (reviewed in Ref. 1). Several cases of complex III deficiency have been reported, some with skeletal muscle symptoms alone (e.g., Refs. 3, 20, 21), one with the defect localized to heart [22]. Cytochrome *c* oxidase deficiencies have also been reported that are tissue specific (e.g., Ref. 23) and in two patients benign or reversible cytochrome *c* oxidase deficiency has been described in infants, implying developmental specificity [24,25].

Diagnoses of mitochondrial myopathies involving the respiratory chain have required the analysis of biopsy tissue. Here we show that respiratory chain defects can be diagnosed by imunoelectron microscopy employing antibodies to the respiratory chain components, in conjunction with protein A-gold as the labeling reagent. This immunolabeling of ultrathin sections was worked out first with bovine heart tissue. Our data are novel in showing the distribution of respiratory chain complexes in the mitochondrial inner membrane in situ (see also Ref. 26). It is clear that the different complexes are randomly distributed in the inner membrane and not aggregated into large patches or islands of protein in the lipid bilayer. Our results do not rule out the existence of smaller functional respiratory chain assemblies containing stoichiometric amounts of the different complexes. These might be seen in a modification of the immunoblotting method used here, by labeling the same thin section with antibody-gold complexes in which gold particles of different sizes are bound directly to the different antibodies.

The patient involved in this study has been shown to have an altered complex III but otherwise normal respiratory chain and no defect in the ATP synthase complex [3]. Electron micrographs of this patient showed abnormal mitochondria with whorls of concentric inner membrane. These mitochondria labeled for complex I and cytochrome *c* oxidase in approximately the same amount as in control human muscle tissue, with

these complexes randomly distributed in the whorled inner membranes. However, there was no significant binding of complex III antibodies, whether anti-holo complex III, core proteins I and II, or Rieske non-heme iron antibody were used, confirming biochemical data in establishing that complex III is not assembled in this patient.

The advantage of being able to analyze for defective respiratory chain components by electron microscopy, as done here, is that only small amounts of tissue are required. The immunogold labeling method can be used on needle biopsy material and where large amounts of tissue cannot be taken, as in small infants. The approach is also a useful adjunct to biochemical methods in that diagnosis of the mitochondrial defect in situ precludes alteration of respiratory chain complexes by proteolysis, or other modifications, during preparation of the tissue for biochemical analysis.

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