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CHRONIC PROGRESSIVE EXTERNAL OPHTHALMOPLEGIA

II. A QUALITATIVE AND QUANTITATIVE ELECTRONMICROSCOPY STUDY OF SKELETAL MUSCLES

ELZA DIAS-TOSTA *

SUMMARY — This study quantifies the major electron microscopic changes in limb muscle biopsies from 31 out of 34 patients with the syndrome of chronic progressive external ophthalmoplegia. Patients were divided into three clinical groups — A) 10 sporadic cases with muscle weakness only; B) 9 familial cases with muscle weakness only; C) 15 cases with muscle weakness and one or more of the following features: pigmentary retinopathy, cerebellar ataxia, pyramidal signs and peripheral neuropathy. Electron microscopic mitochondrial abnormalities were found in all groups (8 patients from group A, 3 from group B, 14 from group C). Quantitative measurements of certain muscle fibre constituents, using a point-counting technique, revealed decreased myofibril volume-fractions and increased volume-fractions of mitochondria, glycogen and lipid in some biopsies from each group. Mitochondrial volume-fractions correlated positively with lipid content, the proportion of type 1 fibres, and the percentage of fibres with increased oxidative enzyme activity. The three groups defined clinically showed no significant differences in terms of the relative proportions of these measured constituents.

Oftalmoplegia externa crônica progressiva: II. Estudo qualitativo e quantitativo por microscopia eletrônica de músculos esqueléticos.

RESUMO — Trata-se de estudo quantitativo das principais alterações encontradas em microscopia eletrônica de biópsia de músculos somáticos de 31 entre 34 pacientes com síndrome de oftalmoplegia externa crônica e progressiva (OECP). Os pacientes foram divididos em três grupos clínicos — A) 10 casos esporádicos, com OECP e fraqueza muscular; B) 9 casos com história familiar positiva, OECP e fraqueza muscular; C) 15 casos com OECP, fraqueza muscular e alguns dos seguintes sintomas: retinopatia pigmentar, ataxia cerebelar, sinais piramidais e neuropatia periférica. Foram encontradas alterações em todos os grupos (8 do grupo A, três do grupo B e 14 do grupo C).

As avaliações quantitativas de certos constituintes das fibras musculares, usando a técnica de contagem de pontos revelou: diminuição da fração-volumétrica de miofibrilas, aumento das frações-volumétricas de mitocôndria, glicogênio e lipídeos. As frações-volumétricas de mitocôndria correlacionam positivamente com o conteúdo lipídico, com a proporção de fibras do tipo 1 e com a percentagem de fibras com aumento da atividade enzimática oxidativa (definidas em estudo anterior). Os três grupos definidos clinicamente não mostraram diferenças significativas em termos de proporções relativas dos constituintes analisados.

Neurocytology Laboratory, National Hospital for Nervous Diseases, University of London, Queen Square, London, England: * M.D., Ph.D.

Unidade de Neurologia, Hospital de Base do Distrito Federal — 70000 Brasília DF — Brasil.

A variety of clinical presentations has been described in association with chronic progressive external ophthalmoplegia (CPEO). According to light microscopy criteria the presence of ragged-red fibres indicates mitochondrial abnormalities. On this basis two groups of patients (A and B) with CPEO were chosen as representing a mitochondrial myopathy, together with another group (C) who did not have a mitochondrial abnormalities [12]. In the latter group the pathological changes found were the "rimmed vacuoles". Electronmicroscopy has demonstrated that the "rimmed vacuoles" were filled with "myelin figures" and other sequestrated products of muscle fibre breakdown [36]. The combined use of histochemical and electronmicroscopy techniques has confirmed the existence of mitochondrial abnormalities both in sporadic and familial cases of CPEO, and also in patients with signs confined to the muscles or with associated signs of other system involvement [2,3,6,21,32,35,40].

These previous studies have shown that the CPEO patients present with at least two groups of myopathological changes, but because of their multiple clinical presentations there are still doubts whether each group represents one single entity or different nosological entities. In an attempt to solve this problem, this study was designed to quantify the subcellular constituents of the muscle fibre from biopsies of patients with CPEO and relate them with the clinical and histochemical data.

MATERIAL AND METHODS

Specimens from 34 patients were studied and 31 of these were suitable for the quantitative study. In each case the muscle sample was taken at resting length using a paired forceps and immediately placed in 3% glutaraldehyde buffered with 0.1M sodium cacodylate (pH 7.3 - 7.4), post-fixed in 1% osmium tetroxide similarly buffered, and embedded in epoxi resin. Sections were cut at 1μm, stained with toluidine blue for 30 sec and examined under the light microscope to select an area with bluish granular appearance. Thin sections (0.06 - 0.09μm) were cut and stained with a saturated solution of uranyl acetate in absolute methanol followed by 0.4% aqueous lead citrate, and examined with a Jeol 120 CX electronmicroscope at 80 KW. To study the variation of the cellular components between the muscle fibres, 20 microscopical fields were sampled in each biopsy; to study the variation within one fibre the cross sectioned muscle fibres were then subsampled in their peripheral and central zones.

Volumetric analysis was performed by the point counting technique. The test system consisted of a grid on transparent acetate paper having a squared network of 144 points, 1.5cm apart. This test grid was placed over the electronmicrographs enlarged to a final magnification of x 28000. Each time one point coincided with mitochondrion, glycogen, lipid or myofibril, one score was made for that muscle fibre constituent. The volume occupied by each element was represented as a percentage of the total volume of the fibre. Because of the preferential zones of distribution of mitochondria in the muscle fibres and I band [13], care was taken always to measure a band of the same depth beneath the muscle plasma membrane. The volumes attributable to mitochondria, myofibrils, glycogen within the sarcoplasm, and lipids were measured individually and the mean relative volumes of these components were calculated together with their standard deviations, variances and standard errors. These data were compared using: 1) one way analysis of variance, to evaluate the distribution of these data in relation to the muscle biopsied, sex of patients and clinical groups; 2) correlation coefficient, to evaluate the relationship between these data and the patient's age when the biopsies were performed, the percentage of fibres with increased oxidative enzyme activity and the proportions of type 1 fibre (the light microscopic data were shown in the previous paper). Correlation coefficients were also applied to compare the relationships between individual components of the muscle fibres.

RESULT

A. Qualitative studies — Twenty-five specimens showed mitochondrial abnormalities; these consisted of paracrystalline inclusions and/or abnormalities in the arrangements of the cristae (Table 1). In 12 cases the intramitochondrial inclusions were abundant (two from group A, two from B and 8 from C) and could be found in mitochondria at the periphery of the fibre, and in those situated more deeply between the myofibrils. Similar but fewer inclusions could be found in 11 other biopsies. Nine cases showed no obvious

Cases	Mitochondria			Lipofuscin	Myelin figures
	Paracrystalline inclusions	Concentric cristae	Abnormal size		
A1	+++	+	+	—	—
A3	+	+	+	+	—
A4	+	—	—	+	+
A7	+	+	—	+	+
A8	+	+	—	+	+
A9	+	+	—	+	—
A10	—	+	+	+	+
A11	+++	+	+	+	—
B4	+++	+	—	+	+
B7	+	—	—	+	+
B10	++	+	+	+	+
C1	+++	+	+	+	+
C2	+++	+	+	—	—
C3	+++	+	+	+	+
C4	+++	+	+	+	—
C6	+++	+	+	+	—
C7	+++	+	+	+	—
C8	+	+	—	+	—
C10	+	+	—	+	+
C11	+	+	—	+	—
C12	—	+	+	+	+
C13	+++	+	+	+	—
C14	+	+	+	+	+
C15	+	+	—	+	—
C16	+++	+	—	+	—

Table 1 — Samples showing mitochondrial changes: +++, abundant paracrystalline inclusions; +, few paracrystalline inclusions; A2, B9, C9, no electronmicroscopy.

changes in mitochondrial ultrastructure. The paracrystalline inclusions consisted of the «intramural type» and the «parking-lot type» (Fig. 1). The inclusions occurred either as a single structure, or in groups linked together by the remaining cristae and matrix, in a longitudinal arrangement (Fig. 2) or in parallel (Fig. 3). The cristae of the mitochondria in twenty-three biopsies showed obvious morphological abnormalities. These included angulated branching of the cristae, the presence of one single concentric crista and multiple layers of concentric cristae (Fig. 2). In the commonest form, the mitochondrion was composed of multiple concentric layers of alternating cristae and matrix. Mitochondria could be found with one single ring-shaped crista, surrounding a rather dense matrix core, or as multiple concentric cristae with either matrix material or matrix and some preserved transverse cristae at the centre (Fig. 2). The combination of concentric cristae and paracrystalline inclusions were seen either in a number of small mitochondria or in one enormously enlarged mitochondrion. On a few occasions elongated paracrystalline inclusion were found closely aligned with the outer mitochondrial membranes. Apart from paracrystalline inclusions, some mitochondria possessed homogeneous electron-dense inclusions, electron-lucent inclusion probably representing lipids, and others contained central aggregations of glycogen granules.

Muscle mitochondria varied greatly in size. Fourteen cases (Table 1) showed a definite increase in mean mitochondrial size. The number of mitochondria was increased in most of the biopsies and this was mainly due to accumulation at the periphery of the fibres. Increased mitochondrial matrix density was a common finding in those specimens showing

other mitochondrial abnormalities (15 out of 25). In general, the abnormal accumulations of mitochondria were subsarcolemmal and peri-nuclear, but agglomerations of these organelles were sometimes seen at the centre of the muscle fibre.

Other abnormalities: *Atrophic fibres* — twenty-eight biopsies possessed either rounded or small angulated fibres, in 8 specimens both forms were represented. The ultrastructure of the small fibre often showed disorganised myofibrils, reduplication of the sarcotubular triadic system and accumulation of lipids. The small fibres usually possessed abnormal folded, or less frequently reduplicated, basement membranes. (Fig. 4). *Focal changes* — seventeen specimens showed streaming of the Z discs, but this was not a prominent feature of any biopsy. In only three cases (1 from group A and 2 from group C) were more profound disarrangements of the internal structure of the fibre seen, such as «cores» (Fig. 5). These

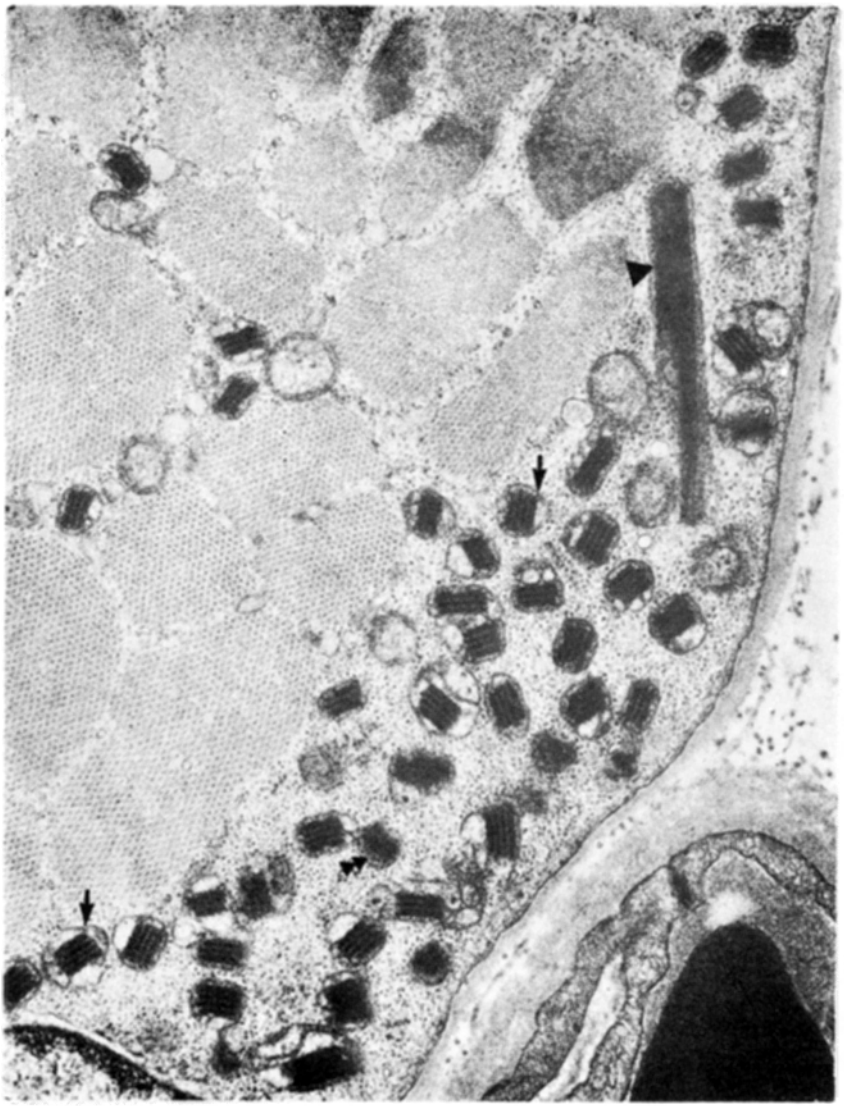

Fig. 1 — A cross section through a collection of subscarcolemmal mitochondria, many of which contain paracrystalline inclusions (arrows), longitudinally cut (arrow head) and obliquely cut (double arrow head) (× 25000).

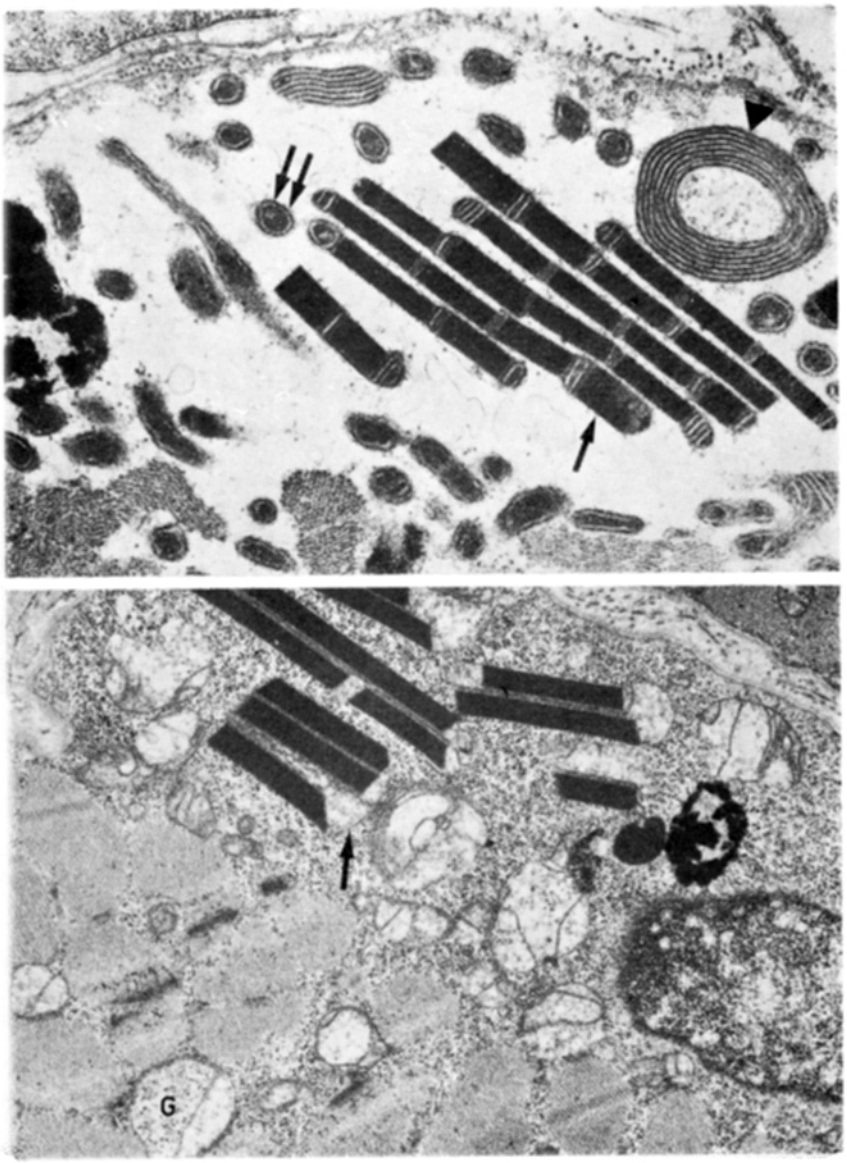

Fig. 2 (above) — Several paracrystalline inclusions of the intermembranal type longitudinally arranged (arrow). To the right a large mitochondrion contains multiple concentric cristae (arrow head). The double arrow shows the increased matrix density (× 21000).

Fig. 3 (below) — Collections of dense paracrystalline inclusions of the intermembranal type, arranged in parallel within a single mitochondrion (arrow). Glycogen granules within the mitochondrion (g) (× 14000).

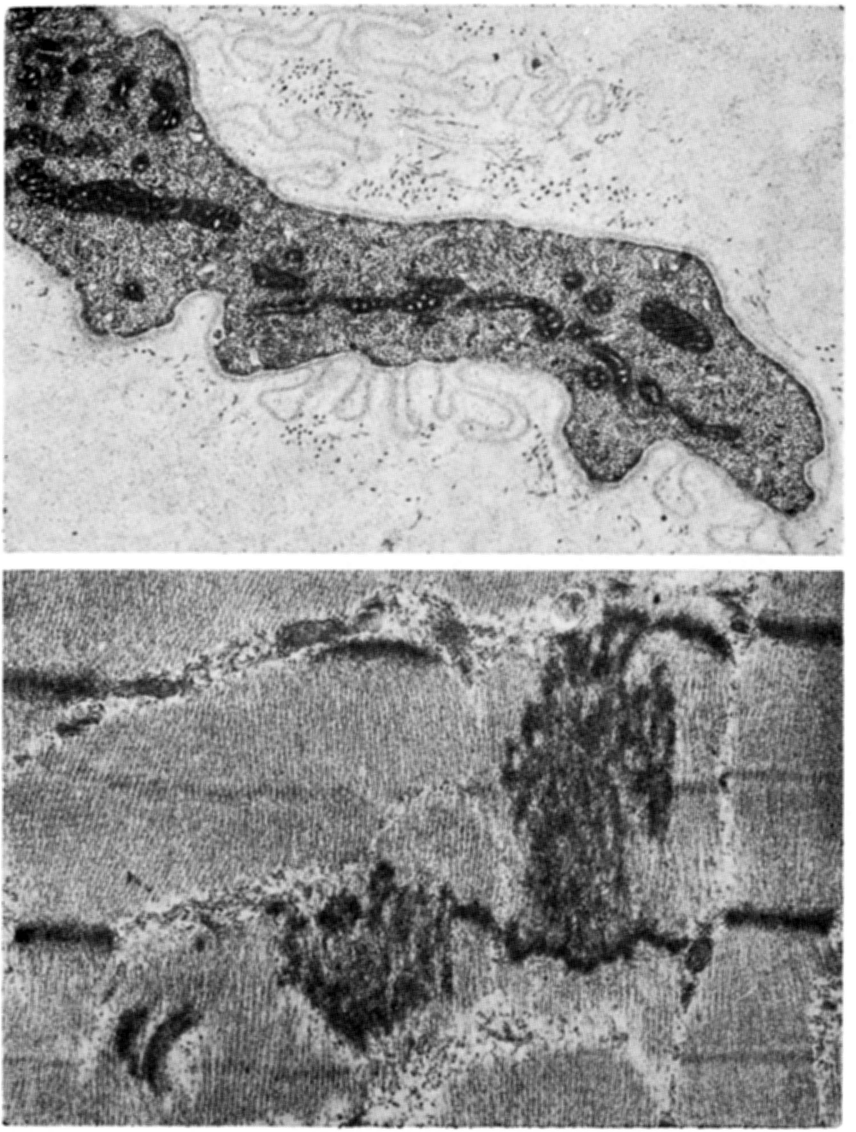

Fig. 4 (above) — A portion of a small atrophic angulated fibre showing marked reduplication and folding of the basement membrane (× 20000).

Fig. 5 (below) — An example of Z line streaming. The Z material extends from one Z line to the next and replaces the normal filamentous of the sarcomere (× 23000).

were longitudinally running zones of local disruption of myofibrillar structure in which the Z discs were difficult to discern; there was a loss of mitochondria and presence of dilated tubules and glycogen amongst the remaining filaments. *Necrosis and phagocytosis* — necrotic myofibres were present in 6 biopsies (three from group A, one from group B and two from group C) but these were never the main features of the disease process, but mast cells were a common feature of the interstitial connective tissue (16 cases). In specimens which showed «rimmed» vacuoles by light microscopy these were filled with variable amounts of cellular debris and «myelin figures». *Satellite cells* were seen in 20 biopsies as a large nucleous surrounded by a narrow rim of cytoplasm. Satellite cells with slight changes from normal morphology were also detected; some contained lipofuscin, others showed non-specific mitochondrial abnormalities, or accumulation of debris between the cell and adjacent muscle fibre. *Other inclusions and structural changes* — lipofuscin was the most common inclusion, found in all but three of the specimens (one from group A and two from group C). In 14 biopsies the sarcotubular system appeared dilated. Tubular aggregates were abundant in one case from group C and occurred sporadically in another 4 (two from group A, one from group B and one from group C). «Honeycomb-like» eleborations of the «T» system and rods were present in one muscle each. «Filamentous bodies» was seen in three specimens and «cylindrical laminated bodies» in another case. *Thickening* of the basal lamina of blood capillaries was a very common observation, either alone or associated with reduplication. A phospholipid inclusion in an otherwise normal-looking endothelial cell mitochondrion was found in one case from group C. Few intramuscular *nerve terminals*, or intrafascicular nerve bundles were found, two of the latter showed a loss of nonmyelinated fibres (two cases from group B). Fig. 6 represents an overall view of the qualitative findings.

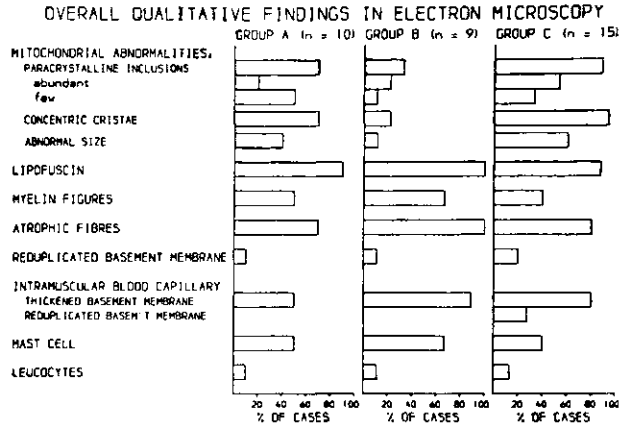

Fig. 6 — Overall qualitative findings in electronmicroscopy.

B. Quantitative studies — Comparison between values from normal controls, measured by other observers (*5,8,16,18*) permitted the establishment of normal limits for the various components: 1) For mitochondrial volume-fraction: Vvm $\leqslant$ 5 normal; marginally increased 5 $<$ Vvm $<$ 7; definitely increase Vvm $\geqslant$ 7. 2) For myofibrillar volume-fractions: Vvmy $>$ 80 normal; decreased Vvmy $\leqslant$ 80. 3) For glycogen volume-fractions: Vvg $\leqslant$ 10 normal; marginally increased 10 $<$ Vvg $<$ 12; definitely increased Vvg $\geqslant$ 12. 4) For lipid volume-fraction: Vvl $\leqslant$ 1 normal; increased Vvl $>$ 1.

In this study the mitochondrial volume-fractions showed a broad range of values (3 to 13%). Of the 31 biopsies measured, 20 (64%) presented one or more abnormal values: 10 (32%) with an increase of mitochondrial volume-fraction, 16 (51%) with a low myofibril content, 10 (32%) with a high glycogen volume-fraction, and 6 (19%) with high values for lipids (Table 2). The most frequent abnormality found was a decrease in myofibril content, the next most frequent being an increase in mitochondria and glycogen. The least affected component was the lipid.

No correlation could be found between mitochondrial volume-fractions, either normal or abnormal, and the sex of the patient, location or degree of weakness of the muscle

Cases	Mitochondria >5%	Mitochondria >7%	Myofibrils <80%	Glycogen-rich sarcoplasm >10%	Glycogen-rich sarcoplasm >12%	Lipids >1
A1	—	+	—	—	—	+
A3	—	+	+	—	+	—
A5	+	—	+	—	+	—
A6	+	—	—	+	—	—
A7	+	—	+	—	+	—
A11	+	—	+	—	+	—
B2	—	—	+	—	+	—
B3	+	—	—	+	—	—
B4	—	+	+	—	—	+
B5	+	—	+	—	+	—
B6	—	+	+	—	—	+
B8	—	—	—	+	—	—
B10	—	+	—	—	—	—
C1	—	+	—	—	—	—
C3	+	—	+	—	+	+
C6	—	+	+	+	—	—
C7	—	+	+	—	+	—
C10	+	—	+	+	—	—
C11	+	—	+	—	+	—
C13	—	+	+	—	—	+
C14	+	—	—	—	—	—
C15	—	+	—	—	—	+
C16	+	—	+	—	+	—

Table 2 — Data on ultrastructural abnormalities from quantitative studies.

biopsied, age of onset of the disease or age at the biopsy, or with any of the clinical groups. However of 5 extensor muscles from the forearm sampled 4 showed an increased mitochondrial content. A direct relationship was found between muscles with type 1 predominance by light microscopy and an increase in mitochondrial volume-fraction. The overall quantitative electronmicroscopy findings are shown in Fig. 7.

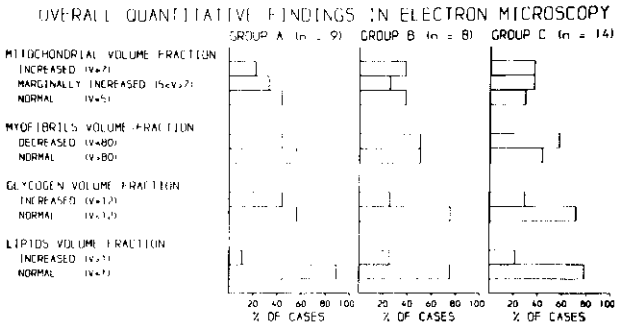

Fig. 7 — Overall quantitative findings in electronmicroscopy.

One way analysis of variance was used to compare the differences in mean volume fractions in different muscles biopsied: left or right side, males or females, and the three clinical groups. None of these factors showed a difference in mean volume-fractions at the 95% level of significance. Using the Pearson correlation coefficient, positive correlations were found when values for the proportion of fibres having increased oxidative enzyme activity at light microscopy (12) were tested against the mean of the total mitochondrial volume-fraction ($p=0.001$), the total volume-fraction of the deep sited mitochondria ($p=0.005$), and the volume-fraction of the superficial mitochondria ($p=0.001$). A negative correlation was found between the proportion of those «ragged-red» fibres and the mean volume-fraction of the total myofibrils ($p=0.02$), and a higher negative correlation with the volume-fraction of the superficial myofibrils ($p=0.003$). The proportion of type 1 fibre at light microscopy (12) correlated positively with the mean of the total mitochondrial volume-fraction ($p = 0.002$), the total lipid volume-fraction ($p = 0.04$), the deeply placed mitochondrial volume-fraction ($p = 0.05$), the superficial mitochondrial volume-fraction ($p = 0.001$) and the superficial lipid volume-fraction ($p = 0.016$) (Table 3). The mean of the total volume-fraction of mitochondria correlated positively with the mean of the total lipid volume-fraction ($p = 0.001$), but negatively with the mean of the total myofibril volume-fraction ($p = 0.17$). The mean of the total myofibril volume-fraction showed a negative correlation with the mean of the total glycogen volume-fraction ($p = 0.001$) (Table 4).

	Volume-fraction	Coefficient	Significance
Proportion of "ragged-red" fibres	Mean total mitochondria	$r = 0.56$	0.001
	Mean total myofibril	$r = -0.43$	0.02
	Mitochondria: deep	$r = 0.50$	0.005
	Mitochondria: superficial	$r = 0.56$	0.001
	Myofibril: superficial	$r = 0.53$	0.003
% of type 1 fibres	Mean total mitochondria	$r = 0.56$	0.002
	Mean total lipid	$r = 0.38$	0.04
	Mitochondria: deep	$r = 0.41$	0.025
	Mitochondria: superficial	$r = 0.58$	0.001
	Lipid: superficial	$r = 0.44$	0.016

Table 3 — Correlation coefficients: light and electronmicroscopy.

	Volume-fraction	Coefficient	Significance
Mean total mitochondria	Mean total lipids	$r = 0.56$	0.001
Mean total mitochondria	Mean total myofibrils	$r = -0.42$	0.017
Mean total myofibril	Mean total glycogen	$r = -0.08$	0.001

Table 4 — Correlation coefficient: proportion of total volume-fractions of the muscle fibre constituents.

COMMENTS

Mitochondrial abnormalities were characterized by the following: the presence of fibres with increased oxidative enzyme activity on histochemistry and increased mitochondrial volume-fraction, abnormalities of mitochondrial size and structure on electronmicroscopy. The presence of paracrystalline inclusions and abnormal arrangements of cristae are the most easily recognisable morphological indices of abnor-

mality of mitochondria [22]. Another criterion used in assessing mitochondrial abnormalities is the alteration in the volume proportions of mitochondria relative to other subcellular components, as determined by quantitative methods of analysis (volume-fraction). The adoption of such methods is made necessary because of the normal variation in the number of mitochondria found in the different sites within individual myofibres and in different muscle fibre types [37,39,42]. Only values greater than one standard deviation above the mean mitochondrial volume-fraction found in normal muscles, used as controls, were regarded as abnormal. No single criterion could be used alone, however, to define the presence of mitochondrial abnormalities. Accumulations of mitochondria as shown by light microscopy as "ragged-red" fibres with the modified Gomori trichrome method or by the increased succinic dehydrogenase activity, have been widely described in muscle biopsies. The abnormal structure of the mitochondria has been shown by electronmicroscopy. These abnormalities have been reported sporadically in cases where there is no reason to suspect a metabolic mitochondrial defect, but they are likely to be due to secondary degenerative changes in the mitochondria. However, in certain conditions there is now good evidence to indicate that changes in mitochondrial structure do reflect an abnormality in one of the pathways of the oxidative metabolism. Some of these cases had already a defined biochemical defect such as in mitochondrial substrate transport [9], mitochondrial substrate utilization [23-25], defect in electron transfer chain [31,33,34,45,47], and a defect of energy conservation and transduction [1,10,14,15,28]. A variety of clinical presentations has been described in association with a biochemical defined mitochondrial defect, including cases of early or late onset myopathy and fatigability, and cases of myopathy and signs of multisystem involvement such as cataracts, growth retardations, cerebellar ataxia, neurosensory loss, pigmentary retinal changes, optic atrophy, mental retardation or regression, and episodic severe metabolic acidosis. In our studies, the mitochondrial morphological abnormalities have been associated with all of different clinical presentations of CPEO. These confirmed earlier studies that showed mitochondrial abnormalities in sporadic and early onset [3,6], sporadic and late onset [38], familiar and early onset with involvement of other organs or systems [17,27,43] and familial and late onset with muscle weakness only [20]. The present data support the concept that the presence of "ragged red" fibres and their ultrastructure counterparts, when they are the maior or exclusive feature in a muscle biopsy from patients with the syndrome of CPEO, indicate mitochondrial myopathy.

Qualitative electronmicroscopy studies confirmed the presence of mitochondrial abnormalities in biopsies containing fibres with increased oxidative enzyme activity characterized on histochemical preparations, and permitted the identification of two further patients having mitochondrial abnormalities in the absence of those fibres. One of these cases was a genetically determined myopathy with vacuolated fibres in light microscope, evident mitochondrial abnormalities was found in his biopsy on electronmicroscope, and further confirmed by the quantitative analysis. Similar case has been reported [20] on qualitative studies. Quantitative electronmicroscopy also revealed increase in the volume-fraction of normal looking mitochondria in another case. The difficulties experienced when attempting to classify patients with CPEO is exemplified by one of our cases, which by clinical, light microscopy, and qualitative electronmicroscopy criteria, appeared to fall within group B, and yet showed a considerable increase in mitochondrial volume-fraction (on quantitative analysis). It may be postulated that the increase in mitochondrial volume-fraction detected is related to the predominance of type 1 fibres in the muscle sampled, but other biopsies having a similar high proportion of type 1 fibre have yielded lower values for mitochondrial volume-fraction. Mitochondrial abnormalities and vacuoles filled with myelin figures may co-exist in the same biopsy indicating that neither is a specific feature of any particular clinical group. While it is accepted that "ragged-red" fibres can present in the muscles of some patients as a non-specific degenerative change [26], this does not explain the increased mitochondrial volume-fraction found in quantitative studies in one of our cases from group B. It is therefore not possible to fix precise limits for the clinico-pathological groups defined earlier, and overlapping cases (one out of group A and four from group B) will occur where more than one criterion is used for classification. Nevertheless groups A and C were homogenous groups on the basis of the qualitative light and electronmicroscopy criteria chosen.

Quantitative analysis of myofibrils, glycogen and lipids showed that usually the myofibrillar component decreased as the proportions of mitochondrial, glycogen or lipids increased. Correlations found between the volume-fractions of mitochondria and lipids strengthened the qualitative finding of increased numbers of lipid droplets

in Sudan black-stained preparations, suggesting the existence of a disturbance of lipid metabolism in the group of patients with an abnormal mitochondrial morphology. It has been pointed out[7] that the abnormal storage of neutral lipids in the muscle fibres of these patients may be an expression of an impaired capacity of the abnormal mitochondria to metabolize neutral fats. The overall increase in glycogen content, while not correlating directly with the increased mitochondrial volume-fractions found, suggests a disturbance of muscle fibre metabolism involving either impaired glycogen breakdown or utilization. Several mild non-specific features of muscle fibre degeneration were found in some cases belonging to all three clinical groups: atrophic fibres (some rounded and others angulated), folded and reduplicated basement membrane and mild dilatation of the sarcotubular system. Only a few biopsies showed a more advanced stage of frank degeneration with necrotic fibres containing macrophages. Although atrophic angulated muscle fibres are usually described as a sign of denervation, no direct evidence of denervation was found in the few intramuscular nerve terminals examined. The significance of the loss of nonmyelinated fibres observed in the intrafascicular nerve bundles of two cases from group B is not known and no loss of sensation or vasomotor regulation was detected in these two cases. Therefore our data do not support the findings of denervation found by other observers[41]. The miscellaneous collections of inclusions found within the myofibres of a number of biopsies were non-specific. The consistent thickening and reduplication of the capillary basal lamina has been reported as a non-specific finding in inflammatory myopathies[19], and in metabolic and degenerative diseases[46]. The findings of reduplication of basal lamina, mast cells and leucocytes may indicate a minor inflammatory component in some stage of this disease, confirming the light microscopy findings in one case reported[4].

The morphological abnormalities seen on light and electronmicroscopy are definitely indicative of at least two different groups of disease mechanisms on patients presenting as chronic progressive external ophthalmoplegia. Although in those patients with positive family story without ragged-red fibres (group B in our study) have been reported nuclear inclusions, said to be characteristic of the disease[29,30,44] the morphological basis are not enough to differenciate between the groups of mitochondrial myopathies[11,12,30]. Each additional step of our morphological analysis showed that different disease entities could be found within CPEO group and it is suggested that further techniques are required to define fully the pathogenic mechanisms in these patients.

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