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MULTIPLE DELETIONS OF THE MITOCHONDRIAL DNA IN POLYMYALGIA RHEUMATICA

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We analyzed the mitochondrial DNA of patients with polymyalgia rheumatica, a disease frequently associated with mitochondrial myopathy. In an attempt to study the deletions, we have developed a qualitative PCR method using a highly thermostable polymerase in order to amplify multiple mitochondrial DNA large fragments (up to 12 kb). PCR serves to observe both deleted and normal fractions of the mitochondrial DNA. We found multiple deletions of the mitochondrial DNA in all of the patient muscles. Although these muscles harbored many ragged red fibers, we found no point mutations of the $tRNA^{Leu(UUR)}$ and the mutation at nucleotide position 8344 was not present.

Polymyalgia rheumatica (PMR) is a clinical entity characterized by stiffness and pain chiefly in the scapular and pelvic girdles, with inflammatory syndrome and prompt response to corticosteroid treatment (1). Some of us (2) found an interesting association between PMR and mitochondrial myopathy, with numerous Ragged Red Fibers (RRF) in the muscle specimens. All tested patients had marked deficiency of the mitochondrial respiratory chain complexes, mainly I and IV (from 37% to 5% of normal activity), that could not be explained by the aging-related OXPHOS defect alone. Moreover the OXPHOS deficit persisted after steroid treatment, although the painful and inflammatory syndromes had disappeared.

PMR has not been reported among the mitochondrial pathologies including an increasing number of nervous and muscular degenerative diseases associated with point mutations, large scale deletions or duplications and depletions of the mitochondrial DNA (mtDNA) (see 3-6 for reviews). RRF have already been shown in diseases that are not attributed to abnormality of mitochondrial function, such as Duchenne dystrophy or polymyositis (7). Nevertheless, to determine whether the mitochondrial abnormalities observed in PMR could be explained by mtDNA mutations, we screened PMR patient muscles for deletions by PCR, and screened for point mutations in $tRNA^{Leu(UUR)}$ and at nucleotide position (np) 8344 by sequencing. The np 8344 mutation is associated with MERRF (Myoclonic Epilepsy and Ragged-Red Fiber) and

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$tRNA^{Leu(UUR)}$ is a gene for which many point mutations have been described and linked to mitochondrial diseases (3).

In an attempt to analyze the deletions, we developed a long PCR allowing detection of both normal and deleted mtDNA fractions. A major limitation of PCR is the size of the amplified product. PCR amplification usually becomes inefficient when the target reaches 5 kb. A few reports of 10 kb target length (8-11) amplifications, and one at 15 kb (12) have been published. These amplifications were performed using highly thermostable polymerases. Improvement has come with the increase of both magnesium concentration and extension time, and with the use of longer primers. Recently great progress was made by Barnes (13) and Cheng (14-15) who respectively amplified targets of 35 kb and 42 kb by using a mixture of two DNA polymerases, one possessing the 3' to 5' exonuclease activity and the other lacking it, but the method seems delicate to make use (16). Until now, most of the large target amplifications have been conducted with pure matrices such as phage lambda or plasmid and very few of them with genomic DNA.

We report here an optimized long PCR amplification which allows one to directly observe both multiple deleted molecules and normal fractions of mtDNA in the muscle of PMR patients. The detection of deletions constitutes one of the first clinical applications of the long PCR.

MATERIALS AND METHODS

DNA purification: Ten specimens from the deltoid muscle of PMR patients (aged 51 to 90 years), 3 young muscle controls and 2 old muscle controls without PMR were analyzed. Genomic DNA was isolated from 10-50 mg skeletal muscle biopsies by standard methods (17).

PCR analysis: PCR analysis was carried out using a highly thermostable Taq DNA polymerase ("GoldStar", Eurogentec, Belgium). The standard PCR conditions were: 1.5 mM $MgCl_2$, 75 mM Tris-HCl (pH 9.0 at 25°C), 20 mM $(NH4)_2SO_4$, 0.01 % (w/v) Tween 20, 1 µM of each primer, 0.2 mM of each dNTP, 500 ng of total DNA as a matrix and 1 unit of GoldStar polymerase in 50 µl reaction mixture. The reaction was conducted using a minicycler (MJR). The cycle times were as follows: denaturation, 30 seconds at 95°C; annealing, 30 seconds at 50°C; and primer extension, 10 minutes at 72°C for 30 cycles. The reaction products were electrophoresed on 0.8% agarose gels at 100 V for 30 minutes. The base sequences and localizations of the oligonucleotide primers used are shown in Table 1.

Sequencing: The region of the $tRNA^{Leu(UUR)}$ and that flanking the np 8344 were amplified by PCR with primers D11-R2 and D6-R5 respectively (Table 1). The purified PCR products were subjected to direct DNA sequencing by using the fmol DNA sequencing system (Promega).

Table 1: Primers used for PCR amplifications and sequencing

Primers	Sequence 5' to 3'	Complementary site	product length
D6	TCTAGAGCCCACTGTAAAG	np 8286 to 8304	
R10	AGTGCATACCGCCAAAAGA	np 382 to 400	8,7 Kb
D5	TCATCGGCGTAAATCTAAC	np 7170 to 7188	
R1	TAGGGTGATAGATTGGTCC	np 2250 to 2268	12 Kb
D11	CCGTAAATGATATCATCTC	np 3200 to 3218	
R2	AATGCTAGGGTGAGTGGTA	np 4169 to 4187	1 Kb
D6	TCTAGAGCCCACTGTAAAG	np 8286 to 8304	
R5	AGGGGGAAATAGAATGATC	np 8602 to 8620	0,3 Kb

RESULTS AND DISCUSSION

$tRNA^{Leu(UUR)}$ seems to be the most subject to mutations (or perhaps the most studied). We failed to detect any point mutations in the sequence of the $tRNA^{Leu(UUR)}$ of four patient muscle mtDNA. In addition, the mtDNA of the four patients showed no MERRF point mutation at np 8344.

The "GoldStar" highly thermostable Taq DNA polymerase allowed us to amplify up to 12 kb of mtDNA sequence with standard PCR conditions from total genomic DNA (Fig. 1). In our hands, the amplifications were efficient and reproducible. This result is probably due to the lack of 3' to 5' exonuclease activity of DNA polymerase and to the optimized buffer. The latter limits the depurination of the template during denaturation because of a high pH. DNA polymerase may be stopped at the depurinations sites created by the acidification occuring during denaturation at high temperature (13). Lastly, the matrix (total DNA) is naturally enriched in mtDNA since there are several thousand mitochondrial genomes per cell, and this is susceptible to improve the efficiency of amplification. Modifications of cycle conditions or addition of glycerol, DMSO or formamide did not improve the quality of the results (data not shown). Over 12 kb, the results were inconstant or less efficient.

Primers D6 and R10 were used to amplify the 8.7 Kb expected mtDNA sequence of the PMR patient muscle so as to visualize some deletions. All the patients harbored multiple deletions (Fig. 2) . The number of deletions varied from 2 to about 15 and the panel was unique and reproducible for each patient. Some deletions were found in several patients, but there was no predominant deletion. No deletion was detected in the young control group (39, 40 and 47 years) and several deletions were present in the old control group (81 and 84 years). Our PCR method makes it possible to amplify the non-deleted fraction of mtDNA and most of the deleted molecules in the same PCR reaction. The technique favors the amplification of the short products but the mechanisms involved are not sufficiently understood to be exploited for quantification. The technique is of interest since the normal molecule does not occult the trace amount of deleted molecules and allows one to visualize some molecules which vary considerably in length.

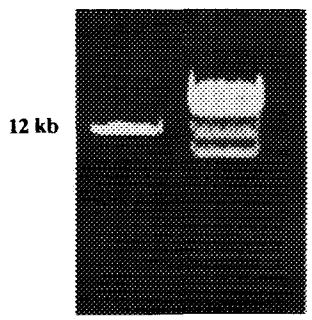

Fig. 1. 12-kb PCR product from mitochondrial DNA, electrophoresed on standard 0.8% agarose gel with lambda DNA *Hind* III as molecular size standard.

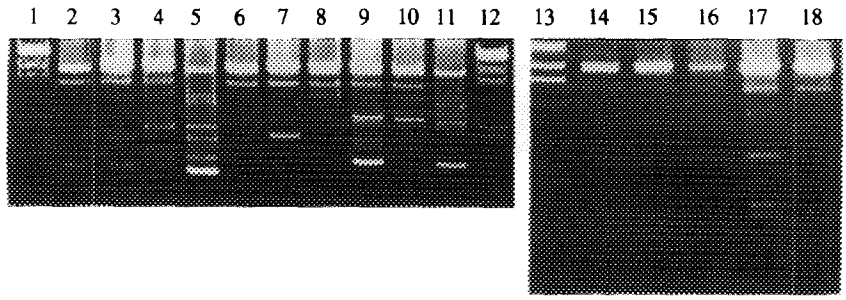

Fig. 2. Electrophoretic migration of the PCR products obtained using primers D6 and R10. Lanes 1, 12 and 13: lambda DNA *Hind* III molecular size marker. Lanes 2 to 11: PCR from muscle DNA of patients with PMR (age: 51, 55, 59, 68, 73, 73, 79, 82, 83 and 90 years). Lanes 14 to 16: PCR from muscle DNA of young patients without PMR. Lanes 17 and 18: PCR from muscle DNA of old patients without PMR.

The specificity of the extra bands was tested and we observed that: (I) Some tissue controls processed in the same conditions had only the non deleted molecules; (II) The increased annealing temperature led to the simultaneous disappearence of the deleted and non deleted forms (Fig.3); (III) The results obtained were reproducible; (IV) The amplification of a 12 kb fragment with primers (D5-R1) situated in external position compared to the D6-R10 primers led to the same pattern for each tissue (Fig.4), which eliminates the possibility of non-specific annealing.

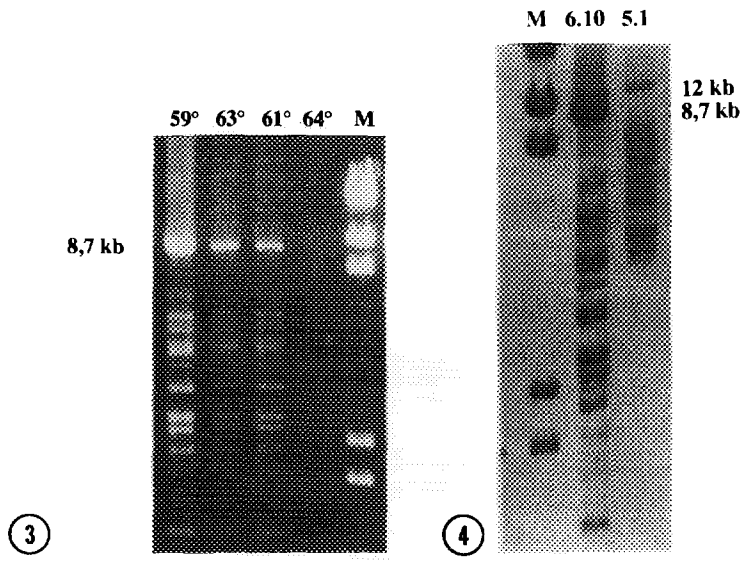

Fig. 3. Simultaneous disappearence of all the amplified fragments when the annealing temperature is increased: The hybridization temperature is indicated at the top of each lane. Lane M: lambda DNA *Hind* III molecular size marker.

Fig. 4. Use of two pairs of primers to amplify the mtDNA of a muscle specimen. 6.10: primers D6 and R10 allowing the amplification of a 8.7-kb fragment. 5.1: primers D5 and R1 allowing the amplification of a 12-kb fragment. M: lambda *Hind* III molecular size marker.

Different kinds of mtDNA deletions exist. Mitochondrial DNA deletions are heteroplasmic and are most commonly searched for and found in clinically affected tissues (3). They vary in size and occur in a region situated between the two replication origins of the mtDNA. A 4977 bp deletion, termed "common deletion" seems to be the most frequently described. In pathology, two types of deletions exist: single deletion which can be visualized by Southern blot method and multiple deletions. In the case of single deletion, the proportion of deleted molecules varies between 20% and 80% of the total mtDNA. These single deletions are found in chronic progressive external ophtalmoplegia (CPEO), in Kearns-Sayre syndrome (KSS) and in Pearson's syndrome (3). On the other hand, accumulation of multiple and varied mtDNA deletions has been suspected during normal aging in the tissues that turn-over slowly (skeletal muscle, heart and brain) (18-26). The rapid turn-over tissues may "eliminate" the abnormal mitochondria and the OXPHOS deficient cells by natural selection. The common deletion has also been shown to accumulate in degenerative diseases such as cardiomyopathies (27) and Parkinson's disease (28). These multiple deletions are not found on Southern blot analysis, because the proportion of each is very low (less than 1%). Indeed, PCR amplification yielded shorter fragments than expected on several occasions (21, 23, 26, 27, 28). In fact the occurence of multiple deletions has probably been seriously underestimated because a given set of primers reveals only one or few deletions when short amplification lenghts are used. Classically, quantifications showed that the maximum amount of common deletion observed in aged muscle was about 0.1 % of total mtDNA. Hence, in this application, PCR is very useful since the deletions could not be observed with Southern blot method.

Current methods vastly underestimate the accumulation of mutated molecules. PCR detection of deleted molecules is very sensitive and allows for a quantitative approach of the mtDNA deleted fraction. This approach has confirmed the accumulation of multiple deletions during life (19, 20). The multiple deletions have been studied by investigating common deletion with the hypothesis that the common deletion / the pool of deletions ratio was constant. Some authors (21, 23, 26, 27, 28) had reported the appearance of a few extra-bands during PCR quantification experiments. Yet, never have so many deletions been observed in the same PCR reaction as in ours. The weak proportion of deleted molecules found with common deletion PCR could not explain any deleterious effect. Two mechanisms may explain the effects of multiple deletions. Firstly, the heteroplasmy must be seen as the coexistence of normal mitochondrial genomes versus a set of mutated molecules. If a great number of deletions or other mutations exist, the addition of multiple mutated molecules could reach a non negligible proportion. Secondly, our PCR method explores an average of a large number of cells and the mutated molecules may be concentrated in certain cells such as RRF. These two mechanisms would better explain how the pathogenic threshold is reached.

The mutational theory of aging held that aging is the result of an accumulation of mutations over time. Mitochondrial DNA seems especially sensitive to this process because of the oxygen radicals present in mitochondria (20). Thus, mtDNA contains higher levels of oxidatively damaged bases than does nuclear DNA and multiple large mtDNA deletions are known to accumulate during aging process (18). Accumulation of damage to mtDNA would be

a major cause of age-related degenerative human disease (19,20). The multiple deletions analyzed by the long PCR method reported here seem to be related to an age factor but this is not sufficient to explain our results. All the same, some young patients with PMR have several deletions that cannot be explained by age (lanes 2 to 5 in Fig. 1). These multiple deletions might be linked to physiological age or be the consequence of a muscle suffering as in PMR.

In summary, we report here the presence of multiple deletions in the muscle of PMR patients and we propose that the accumulation of mutated mtDNA which occurs during life and during degenerative diseases could occur in a more general way when an organ is submitted to a pathologic stress. If the disease affects some non dividing cells, the mutated molecules may not been eliminated and may persist and in turn alter the tissue function. Our long PCR method would be an important tool to explore this hypothesis in other muscular pathologies.

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