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PAPER

Enterovirus related metabolic myopathy: a postviral fatigue syndrome

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Objective: To detect and characterise enterovirus RNA in skeletal muscle from patients with chronic fatigue syndrome (CFS) and to compare efficiency of muscle energy metabolism in enterovirus positive and negative CFS patients.

Methods: Quadriceps muscle biopsy samples from 48 patients with CFS were processed to detect enterovirus RNA by two stage, reverse transcription, nested polymerase chain reaction (RT-NPCR), using enterovirus group specific primer sets. Direct nucleotide sequencing of PCR products was used to characterise the enterovirus. Controls were 29 subjects with normal muscles. On the day of biopsy, each CFS patient undertook a subanaerobic threshold exercise test (SATET). Venous plasma lactate was measured immediately before and after exercise, and 30 minutes after testing. An abnormal lactate response to exercise (SATET+) was defined as an exercise test in which plasma lactate exceeded the upper 99% confidence limits for normal sedentary controls at two or more time points.

Results: Muscle biopsy samples from 20.8% of the CFS patients were positive for enterovirus sequences by RT-NPCR, while all the 29 control samples were negative; 58.3% of the CFS patients had a SATET+ response. Nine of the 10 enterovirus positive cases were among the 28 SATET+ patients (32.1%), compared with only one (5%) of the 20 SATET– patients. PCR products were most closely related to coxsackie B virus.

Conclusions: There is an association between abnormal lactate response to exercise, reflecting impaired muscle energy metabolism, and the presence of enterovirus sequences in muscle in a proportion of CFS patients.

Chronic fatigue syndrome (CFS) is a common disabling condition and a significant public health issue. Recent government sponsored working parties in the United Kingdom have encouraged more research into its origins and treatment.^{1–2} Many CFS patients attribute the onset of their illness to the effects of a previous viral infection. While no association between presumptive viral symptoms and chronic fatigue could be established in one large prospective primary care study,³ chronic fatigue has been found to follow certain specific laboratory confirmed infections, including infectious mononucleosis,⁴ viral hepatitis,⁵ *Coxiella burnetii* (Q fever),⁶ and parvovirus B19.⁷ No such association was found for infection with herpes viruses,⁸ while an association with Borna virus infection is disputed.^{9–10}

Chronic fatigue can also be a sequel of viral meningitis,¹¹ which is most commonly caused by enteroviruses. The coxsackie B serotypes are strongly myotropic but acute infections generally cause only trivial symptoms and rarely result in clinically significant myocarditis or myositis. However, enteroviruses have been implicated in the pathogenesis of chronic myocarditis and dilated cardiomyopathy, and the recent application of the reverse transcription, nested polymerase chain reaction (RT-NPCR), including nucleotide sequencing of PCR products, has unequivocally demonstrated coxsackie B3-like RNA in endomyocardial biopsies from such cases.¹² In contrast, several groups have been unable to find enteroviral RNA in muscle from patients with inflammatory myopathies by RT-PCR,^{13–15} while PCR studies on muscle from CFS patients have given conflicting^{16–17} or negative¹⁸ results.

We showed previously that some CFS patients have abnormal lactate responses to exercise in the subanaerobic threshold exercise test (SATET).¹⁹ This could not be explained on the basis of deconditioning or muscle disuse,²⁰ and subsequently we and others showed that some CFS patients

have abnormal muscle energy metabolism on phosphorus NMR spectroscopy.^{21–23} Fulcher and White provided further evidence of a peripheral component to fatigue in CFS.²⁴ They compared exercise characteristics and muscle strength in CFS patients without concurrent psychiatric disorder, patients with clinical depression (but otherwise having symptoms of CFS), and sedentary controls. While the CFS patients and patients with clinical depression had similar degrees of exercise limitation, the CFS patients were weaker in tests of quadriceps strength.

In the present study, we describe the detection and characterisation of enteroviral RNA in muscle from CFS patients, and examine the relation between the presence of enterovirus and abnormal lactate responses to exercise.

METHODS

Patients and selection of muscle biopsies for analysis

All patients with significant complaints of chronic fatigue and myalgia referred to RJML between 1988 and 1998 were studied using the SATET,²⁵ and also underwent needle muscle biopsy from the left quadriceps on the same day. Portions of each biopsy were retained and stored individually in a vapour phase, liquid nitrogen freezer at -70° in screw capped cryotubes. For the current study, biopsies from 48 cases fulfilling the Oxford criteria²⁶ for the diagnosis of CFS were subsequently selected at random for detection of enteroviral RNA by RT-NPCR.

Abbreviations: CFS, chronic fatigue syndrome; RT-NPCR, reverse transcription, nested polymerase chain reaction; SATET, subanaerobic threshold exercise test

The 48 CFS patients comprised 26 men (mean (SD) age, 35.3 (10.0) years) and 22 women (37.5 (10.8) years) (no significant difference in age). Exercise test findings, muscle histometry, and muscle phosphorus magnetic resonance spectroscopy data from some of these patients have been reported previously.¹⁹⁻²¹

SATET

Lactate responses to exercise at work rates below the predicted anaerobic threshold for each patient were measured as described previously.²⁵ Patients exercised at 90% of the predicted anaerobic threshold work rate (normalised for age, weight, and sex) for 15 minutes. Samples for venous plasma lactate measurements were taken immediately before and after exercise and at 30 minutes postexercise. An abnormal result (SATET+) was defined by a plasma lactate exceeding the previously established upper 99% confidence limit for normal sedentary subjects²⁵ at two or more time points.

Detection and characterisation of enterovirus RNA sequences in muscle

Needle muscle biopsy samples were obtained from vastus lateralis of CFS patients for histological, histochemical, and ultrastructural studies, and for the detection of enteroviral RNA by two stage RT-PCR. Normal tissue for comparison in RT-PCR consisted of 29 muscle samples (three histologically normal biopsies from patients with non-specific leg or arm pain, four samples of abdominal muscle taken at the time of abdominal surgery, six samples of leg muscle taken at the time of amputation in patients without symptoms or signs of neuromuscular disease, and 16 samples from subjects without known muscle disease taken at necropsy examination).

Molecular methods were as described previously.¹² Briefly, frozen tissue samples were homogenised in the presence of vanadyl ribonucleoside complex as an RNase inhibitor, and transfer RNA as a carrier, and nucleic acids were recovered by phenol:chloroform:isoamyl alcohol extraction and ethanol precipitation. Oligonucleotide primers were synthesised on an automated DNA synthesiser (Applied Biosystems, model 381A) using phosphoramidite chemistry.

The enterovirus genomic locations of the primers used and the predicted size of the PCR products are shown in fig 1. RNA was reverse transcribed with SUPERScript II RNase H-free reverse transcriptase (Gibco BRL) in the presence of human placental ribonuclease inhibitor (RNasin; Amersham International) and primer OL68. First stage PCR was done on the reverse transcription mix without further purification, using primers OL252 and OL68 and *Ultma* high fidelity polymerase (Perkin-Elmer). The reagent control for RT-PCR was the successful amplification of the reverse transcribed, processed messenger RNA for glucose-6-phosphate dehydrogenase (G6PD). Tissue samples from normal mice or mice experimentally infected with coxsackie B3 virus were employed sporadically as known negative or positive PCR controls, respectively, as described previously.²⁸

PCR procedures were done in a dedicated suite where preparation of reaction mixes, amplification, and product analysis were conducted in separate laboratories to avoid contamination. Samples of reaction products were visualised after agarose gel electrophoresis and ethidium bromide staining. Second stage nested PCR was undertaken on a portion of the initial PCR reaction product without purification but with the addition of the nested primers OL24 and OL253 and reduction of the template extension time. The second stage PCR product was recovered by isopropanol precipitation and characterised by direct nucleotide sequencing using a double stranded DNA cycle sequencing system (Gibco-BRL) with the same primers as the second round of

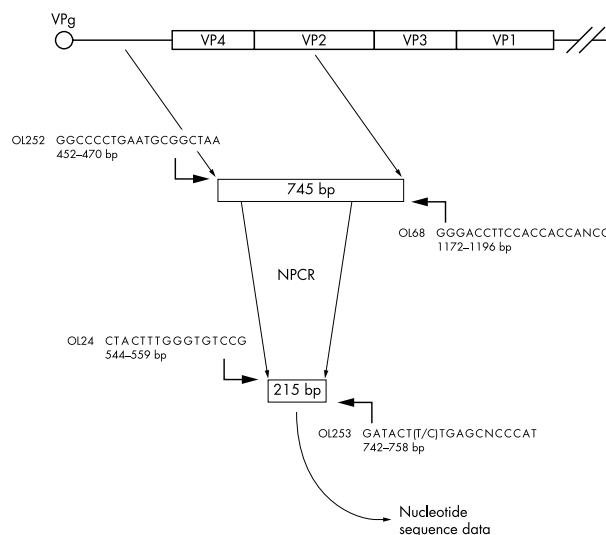


Figure 1 Location and sequence of enterovirus group specific oligonucleotide primers for reverse transcription nested, two stage polymerase chain reaction (RT-NPCR). The second amplicon represents sequences from the 5' non-translated region, immediately upstream of the start site for translation of virus genomic RNA, and contains both conserved and variable sequence motifs. Numbering corresponds to the nucleotide sequence of the prototypal coxsackie virus B3 strain Nancy.²⁷

PCR amplification. Sequencing products were separated by electrophoresis on 6% polyacrylamide gels (SEQUAGEL; National Diagnostics) and the nucleotide sequence was determined in each direction. These sequences were compared with all DNA sequences in the SERC SeqNet database, Daresbury, UK.

We established the fidelity of RT-PCR based, direct nucleotide sequencing by the following control experiments: first, repetitive processing of tissue from a mouse model of experimental infection with coxsackie B3 virus taken at various times (one to six days) after inoculation; second, RT-PCR amplification and nucleotide sequencing of enterovirus serotypes coxsackievirus A9²⁹ and poliovirus 3,³⁰ not previously propagated in this laboratory.

Statistical analysis

The primary aims of this study were to detect enterovirus sequences in muscle from CFS patients and determine the specificity of the observation by comparison with tissue controls (from non-CFS cases); and to examine the relation between abnormal lactate responses to exercise and the presence of enterovirus RNA in muscle.

The number of instances in which enterovirus RNA was detected in CFS cases compared with the tissue controls, and the proportions of enterovirus positive CFS cases among the SATET+ and SATET- CFS cases (the comparison groups), were compared using Fisher's exact probability test.

RESULTS

SATET findings

Twenty eight of the 48 CFS patients (58.3%) were SATET+, having venous plasma lactate levels exceeding the control upper 99% confidence intervals²⁵ at two or more time points. The remainder had normal lactate responses to exercise (table 1).

Detection of enterovirus genomic sequences in muscle biopsy samples

mRNA of the housekeeping gene G6PD was successfully reverse transcribed and amplified from all samples.

Table 1 Lactate responses to exercise in enterovirus positive and negative CFS cases (PCR data from tissue controls for comparison)

	No of SATET+ CFS cases	No of SATET- CFS cases	Tissue controls
PCR+	9	1	0
PCR-	19	19	29

10/48 CFS cases were PCR positive v 0/29 tissue controls (p=0.01). 9/28 SATET+ CFS cases were PCR positive compared with 1/20 SATET- (OR 9.0, 95% CI 1.04 to 78.17). CFS, chronic fatigue syndrome; PCR, polymerase chain reaction; SATET, subanaerobic threshold exercise test; +, positive; -, negative.

Enterovirus positive and normal uninfected mouse tissue controls processed in parallel gave the anticipated result in all cases, indicating no cross contamination between reactions.

Muscle biopsy samples from 10 of the 48 CFS patients (20.8%) were positive for enterovirus sequences, yielding the predicted 215 base pairs (bp) amplicon after two stage RT-NPCR (fig 2). All 29 human tissue controls, while positive for the housekeeping gene G6PD, were negative for enterovirus sequences. This shows a significant association between CFS and the detection of enterovirus sequences in muscle (Fisher's exact probability = 0.011).

Nine of the 10 enterovirus positive cases were among the 28 abnormal responders to SATET (SATET+, 32.1%) compared with only one among the 20 normal exercisers (SATET-, 5%) (table 1, Fisher's exact probability = 0.031). This distribution gives an odds ratio of 9.00 (95% CI 1.04 to 78.17) in favour of an association between abnormal lactate response to exercise and the presence of enterovirus sequences in muscle.

Characterisation of PCR products

The PCR products were characterised by direct nucleotide sequencing. Eight of the 10 amplicons detected in muscle from CFS patients were different from each other. Comparison of these sequences using the SeqNet database showed that in each case the PCR product had greatest homology with the 5' non-coding region of coxsackie B virus genomic RNA immediately upstream of the start site for translation, compatible with the primer design. The sequence variations relative to the prototypal coxsackie virus B3 strain Nancy³⁰ are shown in fig 3. Up to 18 base changes were identified within the 215 bp amplicon. (182 bp within the primers). Two sequences were identical, having two base changes; only one of these cases had an abnormal lactate response to exercise. Three products were identical to this region of the 5' non-coding region of the prototypic CB3 strain Nancy.

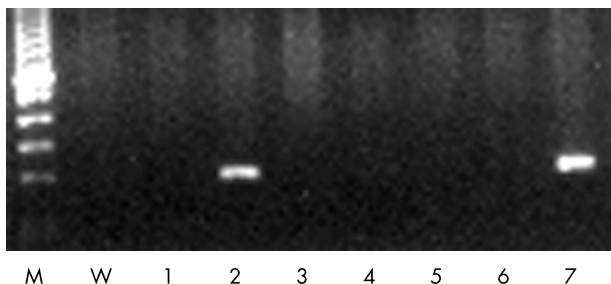


Figure 2 Agarose gel electrophoresis of second stage reverse transcription, nested polymerase chain reaction. The photograph shows the detection of the 215 base pair (bp) product in lanes 2 and 7. M, 100 bp ladder DNA markers; W, water/reagent control; 1-7, samples from CFS cases.

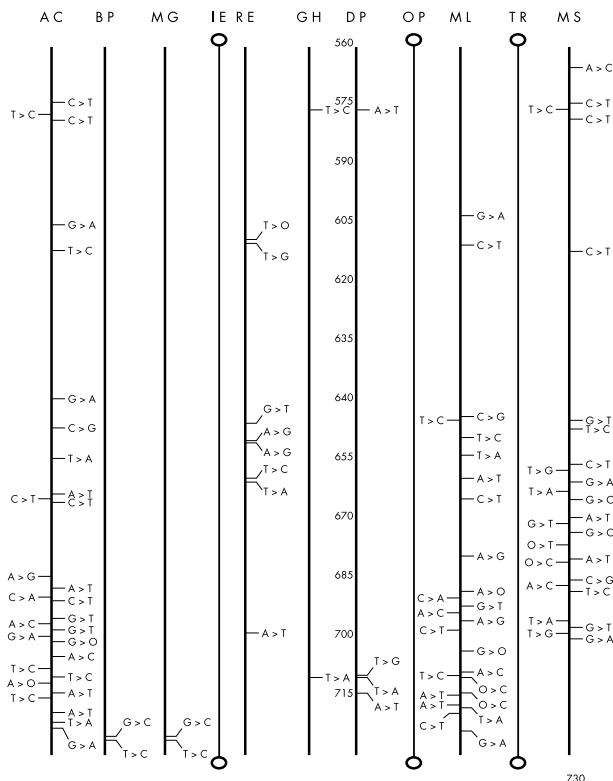


Figure 3 Nucleotide sequences of enterovirus RNA amplified by reverse transcription, nested polymerase chain reaction from muscle of patients with chronic fatigue syndrome. The positions and nature of sequence variations are shown relative to the genomic sequence of the prototypal coxsackie virus B3 strain Nancy (CVB3). Sequences in three cases (IE, GH, and OP) were identical to CVB3 strain Nancy in this region. Two others (BP and MG) were identical to each other.

Fidelity of RT-PCR based sequencing

Repetitive direct nucleotide sequencing in both directions of analogous RT-NPCR products from tissue from a mouse model of experimental coxsackie virus B3 infection or of other enterovirus types established that no sequence changes were introduced by the experimental procedures.³¹

DISCUSSION

Many CFS patients relate the onset of their symptoms to a preceding viral infection from which they believe they failed to recover fully. Evidence supporting such attribution had been largely anecdotal until recent prospective studies found evidence of protracted fatigue states following certain laboratory confirmed infections, supporting the concept of a postviral or postinfectious fatigue syndrome.^{4-7 11}

We reported previously that a subset of CFS patients had abnormal lactate responses to exercise at work rates below the predicted anaerobic threshold.¹⁹ Such cases proved less likely to have evidence of psychiatric disorder than cases with normal lactate responses, and the finding could not be explained satisfactorily by the effects of deconditioning or muscle disuse, either on the basis of heart rate responses to exercise¹⁹ or from a subsequent analysis of muscle fibre sizes and fibre type proportions.²⁰ Further studies using phosphorus magnetic resonance spectroscopy have shown that some CFS patients have defective muscle energy metabolism,²¹⁻²³ notably reduced ATP resynthesis rates following exercise, suggestive of mitochondrial dysfunction.²¹

In the present study, we have correlated abnormal lactate responses to exercise with the detection and characterisation

of enterovirus sequences in muscle, employing techniques used successfully by our group and others to demonstrate such sequences in cardiac muscle.¹²⁻¹³ Enteroviral RNA was detected in 10 of 48 muscle biopsies (20.8%) from our CFS patients but not in 29 samples of control tissue from normal subjects or patients with a variety of muscle diseases, showing that the presence of enterovirus sequences in muscle is not typical of the general population. An abnormal lactate response to exercise was present nine times more commonly in CFS patients with enterovirus sequences in muscle than in enterovirus negative cases.

The mechanism whereby dormant infections might lead to the symptoms of CFS is not clear but chronic activation of the immune system and ongoing cytokine production is one possibility.³² Treatment of malignancies with recombinant cytokines such as interleukins and IFN γ induces symptoms reminiscent of CFS³³⁻³⁴ and increased levels of cytokines including transforming growth factor β (TGF β), INF γ , and tumour necrosis factor α (TNF α) have been reported in plasma in CFS patients.³⁵⁻³⁷ TNF α and IFN γ appear to be key components in cytokine dysregulation in fatigue syndromes which follow both Q fever³⁸ and parvovirus B19 infection.³⁹ Analogous to our observations, studies of patients with post-Q-fever fatigue syndrome revealed *C burnetii* DNA in blood mononuclear cells, liver, and bone marrow several years after primary infection,⁴⁰ and Q fever antigens stimulated aberrant cytokine production in cultured peripheral blood mononuclear cells.³⁸

It must be emphasised that the CFS patients examined in this study may not be representative of the CFS patient population. Many were referred because of prominent muscle symptoms, requiring exclusion of specific neuromuscular diseases. No mechanistic link between defective muscle energy metabolism and enterovirus infection has been established by the present study. Indeed, two patients had identical enteroviral RNA sequence changes but only one had an abnormal lactate response to exercise; mutations at other sites in the viral RNA might therefore determine viral persistence and effects on mitochondrial function. However, the observations presented here support the view that CFS is heterogeneous, and that some cases have a peripheral component to their fatigue related to muscle dysfunction. While the effects of "disuse" and lack of fitness cannot be excluded, previous studies, supported by the evidence presented here, show that some CFS patients have abnormal muscle energy metabolism which is related statistically to the presence of enterovirus sequences in muscle, and that the viruses involved are predominantly coxsackie-B-like. The data support the concept of a true postviral fatigue syndrome following enterovirus infection.

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*Professor L Archard and Dr R Lane designed and coordinated the study. Dr Lane (West London Neurosciences Centre, Charing Cross Hospital) recruited and assessed the patients and performed or supervised the subanaerobic threshold exercise testing, needle muscle biopsies and other investigations. Professor Archard, B Soteriou, and H Zhang (Cell

and Molecular Pathology Group of the Division of Biomedical Sciences, Imperial College Faculty of Medicine) carried out the molecular investigations including reverse transcription, nested polymerase chain reactions, nucleotide sequencing, and comparative sequence analysis on biopsy samples and control tissues.

Competing interests: none declared

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