Investigation by polymerase chain reaction of enteroviral infection in patients with chronic fatigue syndrome

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1. Chronic fatigue syndrome is characterized by muscle fatigue and pain at rest, symptoms which are usually exacerbated with exercise. Although various studies have shown minor, non-specific morphological and biochemical changes in muscle of patients with chronic fatigue syndrome, no consistent defect has been identified. Some have suggested that an enteroviral infection in muscle may cause the chronic muscle fatigue seen in patients with chronic fatigue syndrome, with acute infection directly and irreversibly impairing mitochondrial function, and persistent infection depressing muscle protein synthesis and metabolism.
2. To clarify the involvement of enterovirus infection in chronic fatigue syndrome, muscle biopsies from a group of patients with chronic fatigue syndrome were examined for the presence of enteroviral RNA by reverse transcriptase–polymerase chain reaction techniques in relation to functional studies of muscle mitochondria and the muscle RNA/DNA ratio.
3. Fifty-eight percent of patients reported an uncharacterized 'viral infection' before the onset of their illness, but none of the muscle samples from 34 patients contained detectable amounts of enteroviral RNA. Muscle tissue had a general reduction in the RNA/DNA ratio and mitochondrial enzyme activities with no specific abnormality in the activity of enzymes encoded partially on the mitochondrial genome (cytochrome-*c* oxidase) or nuclear genome (citrate synthase, succinate reductase).
4. These data provide no evidence of an enteroviral infection in muscle of patients with chronic fatigue syndrome, although this does not exclude a role of enterovirus in initiating the disease process. The general reduction in RNA/DNA ratio and mitochondrial enzyme activities is consistent with a general reduction in habitual activity.

INTRODUCTION

Chronic fatigue syndrome (CFS) is a debilitating illness which can affect a significant number of people. The pathophysiology of CFS remains unknown and clinical symptoms are non-specific, although the illness is characterized by fatigue at rest which may be exacerbated by exercise [1].

Analyses of muscles from patients with CFS have produced diverse and confusing results. Studies by Arnold et al. [2] demonstrated an abnormality in muscle metabolism in a single patient with myalgic encephalomyelitis, after an infection with varicella-zoster. However, these features were not common in other patients with myalgic encephalomyelitis, but were consistent with NMR findings from muscles of more sedentary subjects. Further studies have found no consistent metabolic abnormalities in patients with CFS [3, 4].

Analyses of muscles from patients with CFS have demonstrated a range of minor, non-specific morphological changes, although there appeared to be no consistent correlation between clinical symptoms and any of the histological abnormalities observed [1, 5]. More dramatic histological abnormalities have been reported by others [6].

Biochemical abnormalities have been reported in muscles from patients with CFS. RNA content of quadriceps muscle biopsies from these patients has been shown to be significantly reduced [7], suggesting that the capacity of the muscle to synthesize protein was reduced. However, this had not resulted in a loss of muscle bulk since muscle protein content was maintained.

The possible relationship between viral infections and CFS has received considerable attention over the last few years, although the mechanism by which a viral infection might induce the symptoms of chronic fatigue remains unclear. Patients frequently report that the onset of the illness follows an apparent 'viral infection' although this is rarely proven [8]. Numerous viruses which have been implicated in this role include herpesviridae [9–11], retroviruses [12] and enteroviruses [13–15]. However, this has recently been disputed in a number of studies which found no evidence for an involvement of these viruses in CFS [16–20].

Key words: chronic fatigue syndrome, creatine kinase, mitochondria, muscle pain, polymerase chain reaction, virus.
Abbreviations: CFS, chronic fatigue syndrome; PCR, polymerase chain reaction.
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A role for enterovirus infection in the initiation and persistent nature of CFS appears more likely, although published data are confusing. Gow et al. [21] reported initially that a higher proportion of muscle biopsies from CFS patients contained enteroviral RNA sequences, detectable by polymerase chain reaction (PCR), compared with control samples. Furthermore, whole virus particles were visualized by electron microscopy in the positive samples [22]. Preedy et al. [7] reported that about 80% of patients with CFS had serology indicative of persistent enteroviral infection, and Bowles et al. [15] used slot-blot hybridization techniques to demonstrate the presence of enteroviral RNA in a significant number of cases of CFS compared with control muscle samples. More recently, Clements' group [23, 24] have demonstrated the presence of novel enteroviral species in the serum of a significant number of patients with CFS. In contrast, Swanink et al. [25] found only one positive sample from a group of 75 patients with CFS and 75 controls by serological testing, VP-1 antigen testing or PCR analysis of stool samples.

Gow et al. [26] have reported a further, larger study of enteroviral RNA in patients with CFS and found no increased incidence of enteroviral infection in muscles of their patient group compared with a control group. This is in contrast to their preliminary report [21] and these authors have now proposed that an acute, short-lived viral insult predisposes mutant or defective mitochondria to selectively proliferate, resulting in an impaired mitochondrial respiration. This mitochondrial impairment persists with subsequent muscle fatigue upon exertion and thus leads to the symptoms of CFS. In support of this a significant number of the patients with CFS examined were reported to have mitochondria with morphological changes, hyperplasia, hypertrophy, pleomorphism and proliferation of mitochondrial cristae [6, 26]. However, the relevance of this finding was not supported by functional mitochondrial measurements. Whether levels of enteroviral infection which can only be detected by PCR or 'nested PCR' techniques could cause the diffuse, widespread symptoms of CFS remains unclear.

The aim of this study was to examine the possible involvement of enteroviral infection in muscles from patients with CFS and the relationship of this to reported mitochondrial abnormalities in muscles of patients with CFS. The PCR techniques described by Gow et al. [21] were used to search for the presence of enteroviral RNA. The activities of a range of mitochondrial enzymes encoded for on both the nuclear and mitochondrial genome were measured as described by Wagenmakers et al. [27], and muscle RNA/DNA ratios were also determined. Serum creatine kinase activity was routinely measured as an index of muscle damage in the patients.

METHODS

Patients

Patients were diagnosed as having CFS on the basis of their complaints of muscle pain and fatigue. The diagnosis conformed to the Oxford Consensus Criteria [28]. Patients who consented to muscle biopsy were randomly selected for the study from the population attending the Chronic Fatigue Clinic at the Royal Liverpool University Hospital. Local ethical approval was granted for the study and informed consent was obtained from the patients. Muscle biopsy samples were taken from 54 patients with CFS who were recruited from over 450 patients seen in the clinic over an 18 month period.

Ten control samples were obtained from patients who were undergoing routine orthopaedic surgical procedures. These control subjects showed no clinical or biochemical evidence of muscle damage or fatigue.

Plasma creatine kinase activities were determined by the Department of Clinical Chemistry, Royal Liverpool University Hospital.

Measurement of muscle enzyme activities

Muscle biopsy samples of approximately 200 mg were removed routinely from the anterior tibialis muscle using the conchotome technique under local anaesthesia [29].

The activities of mitochondrial enzymes coded for on both the nuclear genome (citrate synthase, succinate reductase) and partially on the mitochondrial genome (cytochrome-*c* oxidase) were measured as described previously [27].

Determination of muscle RNA/DNA ratio

Between 10 and 20 mg of muscle was ground under liquid nitrogen and sonicated in 1 ml of 2% perchloric acid. The sample was centrifuged at 4500 *g* (Sorval RC-5 Superspeed Refrigerated Centrifuge, GSA fixed rotor) for 30 min at +4°C and the pellet was washed twice and re-centrifuged. The resulting pellet was digested with 2 ml of 0.3 mol/l sodium hydroxide at 37°C for 1 h. A 0.1 ml aliquot was removed for subsequent protein assay [30]. The remaining solution was neutralized with 60% perchloric acid and re-centrifuged at 4500 *g* for 30 min at +4 C. The supernatant was retained and the pellet washed in 2% perchloric acid and re-centrifuged. Supernatants were combined and RNA concentration was determined using the dual wavelength method described by Ashford and Pain [31]. DNA content of the pellet was determined by the method of Cocrane et al. [32].

Detection of enteroviral RNA

Thirty-four muscle samples from patients with CFS and 10 control samples were analysed for the presence of enteroviral RNA.

Muscle samples were powdered under liquid nitrogen in a mortar and pestle and sonicated in guanidinium thiocyanate. Total muscle RNA was extracted from the biopsy sample by the method of Chomczynski and Sacchi [33].

RNA was extracted from coxsackie B5 or Echo 11 virus infected Vero cell lines in a similar manner. Medium was removed from a confluent flask of cells. Cells were washed twice with PBS and solubilized directly in guanidinium thiocyanate solution. The resulting solution was sonicated and RNA extracted as described for muscle samples.

In order to ensure that the RNA extracted from the infected cells and muscle biopsy samples was intact, the samples were initially analysed for the presence of Ableson tyrosine kinase mRNA which is known to be expressed in human muscle [21, 34].

Reverse transcriptase–PCR was undertaken using the Perkin–Elmer Thermostable rTth Reverse Transcriptase RNA PCR kit (Perkin–Elmer Cetus, Beaconsfield, U.K.) in the presence of the upstream primer ABL2 at a temperature of 70°C for 15 min.

A programmable thermal cycler (Omnigene; Hybaid, Teddington, U.K.) was used for DNA amplification. Amplification of Ableson tyrosine kinase mRNA was carried out in 35 cycles, where one cycle consisted of 1 min at a melting temperature of 94°C, 1 min at an annealing temperature of 55°C and 1.5 min at an elongation temperature of 72°C in the presence of primers ABL1 and ABL2 shown below. DNA products were analysed by agarose gel electrophoresis through a 2% gel containing 0.2 μg/ml ethidium bromide for 1 h at 100 V in Tris/borate/EDTA buffer at pH 8.2. Gels were visualized under UV trans-illumination.

ABL1: 5′ CAG CGG CCA GTA GCA TCT GAC TT 3′

ABL2: 5′ TGT GAT TAT AGC CTA AGA CCC GGA G 3′

The PCR primers chosen to amplify tyrosine kinase [34] give rise to an amplified sequence of 218 bp. Samples which produced a positive amplification of this mRNA were subsequently analysed for enteroviral RNA. An enteroviral infected cell line positive control sample was included in each amplification run.

Reverse transcriptase–PCR conditions were optimized using a Vero cell line infected with coxsackie B5 or Echo 11 virus (obtained from the Department of Medical Microbiology, University of Liverpool). PCR sensitivity was determined by analysis of serial dilutions of RNA extracted from infected cell lines, the end point being the highest dilution of sample producing a visible product when analysed by agarose gel electrophoresis as described previously. A non-infected cell line was used as control. Reverse transcriptase–PCR was undertaken using the Perkin–Elmer Thermostable rTth Reverse Transcriptase RNA PCR kit (Perkin–Elmer Cetus) with an optimal magnesium concentration of 0.8 mmol/l. The EP1/EP4 enteroviral PCR primers (Molecular Medicine Unit, King's College School of Medicine and Dentistry, Rayne Institute, London) employed by Gow et al. [21] were used. These encoded a sequence common to a range of enteroviruses [21] and spanned an area of 414 bp.

EP1: 5′ CGG TAC CTT TGT GCG CCT GT 3′ (base pairs 64 to 83)

EP4: 5′ TTA GGA TTA GCC GCA TTC AG 3′ (base pairs 459 to 478)

DNA amplification was carried out in 35 cycles. One cycle consisted of 1 min at a melting temperature of 94°C, 1 min at an annealing temperature of 50°C and 1.5 min at an elongation temperature of 72°C.

The statistical significance of results was assessed by Student's *t*-test, a P value of less than 0.05 being considered significant.

RESULTS

Clinical details

The average age of the group of patients studied was 38 ± 2.2 years (range 22.9–54.4 years), and 63% were female. These were typical of the large population of patients attending the clinic (450 patients, mean age 40.9 ± 12.9 years, range 15.4–83.5 years, 62% female). Questionnaire analysis showed that 58.3% of the patients had reported a 'viral infection' before the onset of their illness although none had been clearly characterized at that time. Seventy-six percent of patients complained of muscle pain and 65% of a sleep disturbance. Interestingly, the majority (61%) of those without muscle pain had no sleep disturbance. Analysis of serum creatine kinase activities revealed no evidence of widespread severe muscle damage, but comparison of patients with muscle pain and those who have never reported pain indicates that some subjects may have evidence of minor levels of muscle damage leading to marginally elevated plasma creatine kinase activity (Fig. 1).

Muscle biopsy samples from patients with CFS had a general reduction in all of the mitochondrial enzyme activities measured, in comparison with values for control subjects previously reported by our group (Table 1). There was no different pattern

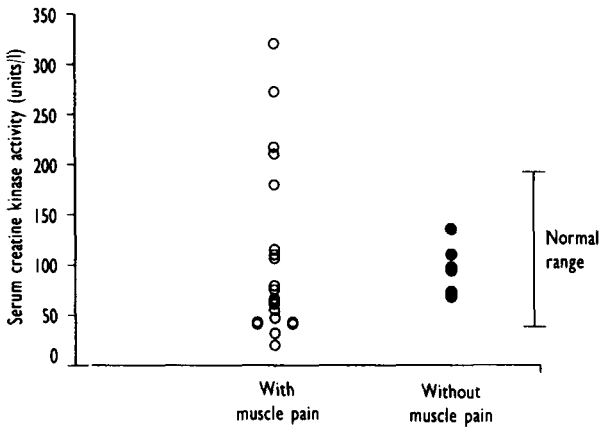

Fig. 1. Serum creatine kinase activities of patients with CFS with or without muscle pain

between those enzymes coded for on the nuclear or mitochondrial genome. Patients with CFS had a large reduction in the muscle RNA/DNA ratio compared with control values (Fig. 2*a*), although there was no difference in protein content between patient samples and controls (Fig. 2*b*).

Forty (31 patients, nine controls) of the 44 (34 patients and 10 controls) muscle samples analysed were found to contain mRNA for tyrosine kinase (Fig. 3*a*) and were therefore used for analysis for enteroviral RNA. Enteroviral RNA was detected in total RNA samples from coxsackie B5 and Echo 11 virus infected cell lines (Fig. 3*b*). Two microgrammes of total RNA extracted from cell lines was serially diluted. Enteroviral RNA was detected at a dilution of 1×10^{-6} and above (results not shown in detail). Analyses of RNA of all muscle biopsy samples previously shown to contain tyrosine kinase mRNA failed to detect the presence of any enteroviral RNA (Fig. 3*b*).

DISCUSSION

The results presented here provide no evidence that patients with CFS have a persistent enteroviral infection in muscle. This does not appear to be due to lack of sensitivity since the PCR analysis of enterovirus infected cell lines was successful in detecting low levels of enteroviral RNA. It is difficult to compare accurately the sensitivity of PCR techniques between research groups based on RNA extracted from infected cell lines, since this would depend on the level of infection in the cells. However, analysis of sequential dilutions of RNA extracted from cell lines appeared to demonstrate a similar sensitivity to that described by Gow et al. [21].

Our data do not agree with those of Gow et al. [21] for either patients with CFS or control subjects. These authors reported that 15% of control samples contained enteroviral RNA sequences, in contrast to our study and those of others [35–37]. Fox et al. [35], Leon-Monzon and Dalakas [36] and Jongen et al. [37] used PCR techniques with PCR primers also aimed at the 5′ non-coding region of a range of enteroviral species to examine muscle samples from patients with inflammatory myopathies for the presence of enterovirus. None of the groups found any evidence of virus in over 150 patients or control samples. In contrast, Behan and Behan [22] found that 56% of samples from patients with inflammatory myopathies contained enteroviral RNA. Fox et al. [35] suggest that the differences between studies are likely to reflect differences in the nature of the samples used since the control samples which contained enteroviral RNA in the study of Gow et al. [21] were from patients with malignant tumours. In the same study [21], no healthy young adult had a positive result.

The difference in findings between our study and that of Gow et al. [21] may be due to the population of CFS patients studied. Although the diagnostic criteria of both groups was broadly similar, the patients used in the study of Gow et al. [21] all reported that the illness had an acute onset after a feverish illness, whereas only 58% of patients in our study reported a viral infection before the onset of their illness.

Another possible explanation comes from the work of Galbraith et al. [24]. Direct sequencing of PCR products from enterovirus-positive serum samples of patients with CFS by these authors has suggested the presence of distinct novel enteroviruses. These viruses are thus unclassified and their site of replication is unknown. Thus, the different findings of different studies may reflect the nature of the samples examined. Alternatively, it may be that these novel viruses replicate in non-muscle cells such as macrophages and that infiltration of damaged muscle by macrophages is a necessary prerequisite for the detection of viral presence.

Data presented here provided no evidence of a primary defect in muscle mitochondrial function. This is in keeping with the report of Barnes et al. [4] who described no gross abnormalities in mitochondrial function in patients with CFS using NMR spectroscopy.

The reduced RNA/DNA ratios reported here (Fig. 2*a*) indicate a reduced capacity for muscle to synthesize protein, although muscle protein degradation must be comparably reduced since muscle protein content is maintained (Fig. 2*b*). These data and previous studies by others [7, 27, 38] have therefore demonstrated a general reduction in both mitochondrial enzyme activities and RNA/DNA ratios in muscles of patients with CFS. A likely explanation is that these may be secondary consequences of reduced habitual activity, which is universal in patients with CFS. It has previously been hypothesized by our group [27] that a 'vicious cycle' of biochemical changes occurs in patients with CFS after a variety of possible initial 'insults' which may include a mental depression, or debilitating

Table 1. Activity of mitochondrial enzymes in muscle samples from patients with CFS. Results are expressed as mean ± SEM, $n = 54$ for CFS group, $n = 16$ for control group. Control values have been published by our group previously [27]. Statistical significance: $^*P < 0.05$.

	Patients with CFS	Control subjects
Cytochrome-c oxidase (μmol min^{-1} g^{-1} of wet wt.)	33.6 ± 3.4	50 ± 2.4*
Succinate reductase (μmol min^{-1} g^{-1} of wet wt.)	3.2 ± 0.4	7.7 ± 1.5*
Citrate synthase (μmol min^{-1} g^{-1} of wet wt.)	10.3 ± 0.9	13.7 ± 0.9*

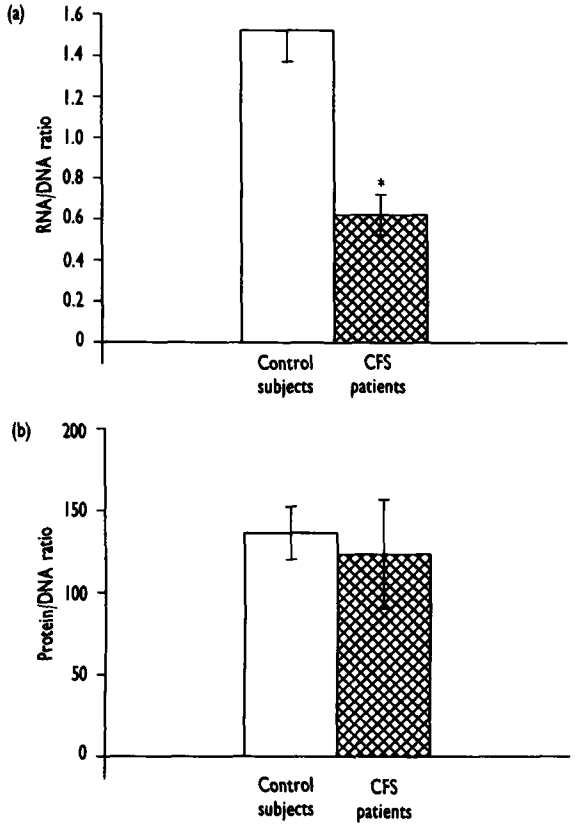

Fig. 2. RNA/DNA ratio (*a*) and protein/DNA ratio (*b*) in muscle biopsy samples from control subjects and patients with CFS. Statistical significance: $^*P < 0.05$.

viral infection. This 'insult' leads to inactivity with ensuing reduction in muscle mitochondrial enzyme activities. This would result in an exercise intolerance, muscle pain on exercise and hence further inactivity etc. The data presented here are fully compatible with this theory.

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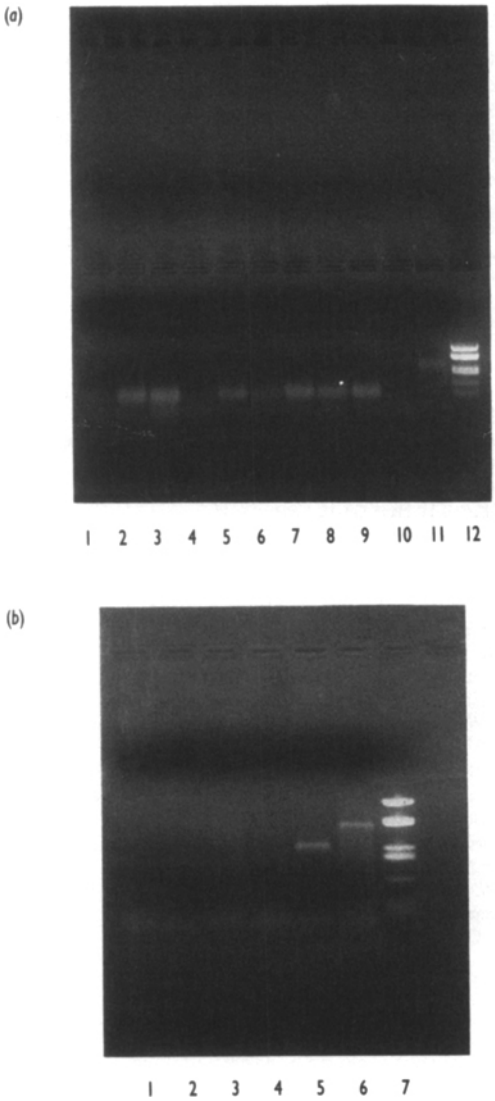

Fig. 3. Amplification products of RNA isolated from muscle biopsy specimens of patients with CFS. (*a*) Lanes 1–9 contain muscle biopsy samples from patients with CFS amplified with ABL gene primers. Lane 11 contains the 308 bp positive control PCR product from the Perkin–Elmer RNA PCR kit. Lane 12 contains base pair markers. (*b*) Lanes 1–4 contain muscle biopsy samples from patients with CFS amplified with enteroviral primers EP1 and EP4. Lane 5 contains the 308 bp positive control PCR product from the Perkin–Elmer RNA PCR kit. Lane 6 contains the PCR product of enteroviral infected Vero cells. Lane 7 contains base pair markers.

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