

Studies on Enterovirus in Patients with Chronic Fatigue Syndrome

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A large study on 121 patients with the chronic fatigue syndrome (CFS) that examined muscle biopsy samples for enterovirus by means of polymerase chain reaction analysis was carried out. The results were compared with those obtained from 101 muscle biopsy specimens from patients with a variety of other neuromuscular disorders (OND), including neurogenic atrophies, dystrophies, and mitochondrial, metabolic, and endocrine myopathies. Thirty-two (26.4%) of the biopsy specimens from the group of patients with CFS were positive, compared with 20 (19.8%) from the group of patients with OND, a difference that was not significant. This finding is in contrast to those of our previous smaller study in which significantly more patients with CFS than control subjects (53% [32 of 60] vs. 15% [6 of 41]) had enterovirus RNA sequences in their muscle. It was concluded that it is unlikely that persistent enterovirus infection plays a pathogenic role in CFS, although an effect in initiating the disease process cannot be excluded.

The chronic fatigue syndrome (CFS), also known as post-viral fatigue syndrome or chronic fatigue and immune dysfunction syndrome, is an unusual disease of unknown etiology. Investigation into this disorder has been difficult because of the lack of objective clinical findings. Strict diagnosis criteria, however, have now been discussed widely and established [1–4]. The core symptoms are severe fatigue made worse by exertion and myalgia. These are accompanied by a variety of psychiatric and other symptoms [2, 5]. The illness usually follows an acute viral-like infection and may occur sporadically or in epidemics [5, 6]. In the latter, the symptoms may originally suggest poliomyelitis [7]; indeed, older nomenclature for the disorder often included poliomyelitis in the title (e.g., abortive poliomyelitis, atypical poliomyelitis, and encephalitis resembling poliomyelitis) [5]. Antibody studies have also raised the possibility of a poliomyelitis-like virus, as in one of the largest and best documented epidemics in Akureyri, Iceland [5, 8]. Other epidemiological reports, however, have indicated a role for different enteroviruses [5]. In spite of the evidence for an infectious agent, all attempts at routine culture of virus in epidemics have failed [5].

Serological studies of small outbreaks in Scotland showed increased titers of antibody to coxsackieviruses in the patients [9]. The data, however, were difficult to interpret because they related to specific IgG, rather than IgM, antibody.

In two more recent studies, no difference in titers of specific antibody to coxsackievirus was detectable between patients and controls [10, 11].

Direct isolation of enteroviruses from patients with CFS has been attempted [12]. Concentrated fecal samples from 76 patients with CFS and 30 matched controls were cultured, and positive results were obtained for 22% of patients, compared with 7% of controls. In addition, enterovirus antigen (VPI polypeptide) was detected in the circulation, either free or complexed with antibodies, together with specific IgM antibody in 51% of patients [12]. One year later, up to 53% of this group of patients were still seropositive for VPI antigen. These results indicate an association between chronic infection with enteroviruses and CFS [12].

Following these studies, the natural progression was to use molecular biological techniques in a search for enterovirus sequences. Quantitative slot-blot technology and complementary DNA probes for coxsackievirus, used in a study of muscle biopsy samples from 76 patients with CFS, revealed that 20% showed a positive hybridization signal compared with none of the controls [13]. There is a nonspecific background effect in this method, however, and the sensitive and specific polymerase chain reaction (PCR) was next used in a carefully controlled study of 60 patients compared with 41 controls [11]. Overall, significantly more patients than controls had enterovirus RNA sequences (53% vs. 15%), thus indicating that persistent enterovirus infection of muscle may occur in some patients with CFS and may have a pathological role.

The question of whether a defective virus is present in patients with CFS was raised by Cunningham and co-workers [14]. They prepared enterovirus group-specific RNA probes complementary to either the positive (genomic) or negative (template) strand of enterovirus RNA and used these riboprobes to compare replication of virus in the muscle of patients with CFS with that in an acute infection with coxsackievirus B2 in tissue culture. In the acute infection, positive-strand RNA was synthesized in ~100-fold excess

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compared with the negative-strand RNA. In contrast, in the patients' muscles, the amount of positive and negative strands of enterovirus RNA was approximately equal. It was suggested, therefore, that persistence of enterovirus in muscle might be due to a defect in the control of viral RNA synthesis [14].

We have extended our initial work using the PCR [11] to search for enterovirus sequences in the muscle biopsy specimens from patients with CFS to investigate a further 121 cases. The results are reported here.

Patients and Methods

Selection of CSF Cases

One hundred twenty-one patients with CFS, who were admitted consecutively to the Institute of Neurological Sciences (Glasgow, Scotland) during a 12-month period, were examined. There were 49 men (age, 18–60 years; mean, 41.2 years) and 72 women (age, 18–52 years; mean, 37.7 years). The criteria for selection were as previously described [1, 2], i.e., onset of severe fatigue after an acute, possibly viral, episode that lasted >1 year and was severe enough to reduce daily activity to <50% of the premorbid level. Symptoms included overwhelming fatigue made worse by exercise, myalgia and depression, poor concentration, and loss of short-term memory. The severity of the disease fluctuated with occasional short periods of remission. All patients had considered themselves to be in good physical health with satisfactory work and social records prior to their illness. They underwent detailed investigation, as previously described [11], to exclude any other cause for their complaints.

Selection of Cases of Other Neuromuscular Diseases

Forty men aged 19–68 years (mean, 54 years) and 61 women aged 17–70 years (mean, 49.6 years) who were undergoing diagnostic muscle biopsies were included. These patients were from the same catchment area as the patients with CFS and were investigated over the same period and at the same hospital, and the same muscle site was examined, i.e., musculi vastus lateralis. Their diagnoses were metabolic, endocrine, mitochondrial, alcoholic, and neurogenic myopathies and various dystrophies.

Muscle Biopsies

Three cores of skeletal muscle were taken from the right or left vastus lateralis with the modified UCH needle under local anesthesia. One and one-half cores were snap frozen in cooled Arcton (ICI, Glasgow) or liquid nitrogen for storage and examination by histochemical and routine staining, and one-half core was fixed in 2% glutaraldehyde for ultrastructural analysis. The core for examination by the PCR was

retrieved from the cannula with a sterile needle, placed in a sterile container, snap frozen, and stored in liquid nitrogen until required.

Hybridization Methods

Polymerase chain reaction. RNA was prepared from muscle biopsy specimens, and the PCR was carried out as previously described [11]. The quality of the RNA from the biopsy specimen was ensured by visualization of the 28S and 18S bands on agarose gels and by the amplification of the Abelson (*abl*) tyrosine kinase “housekeeping” gene that gives rise to a product of 218 base pairs [15]. The PCR primers for enterovirus that have been previously described were used [11]. Great care was taken to avoid false-positive results due to contamination. Dedicated positive displacement pipettes were used, uninfected RNA from human tissue cell cultures was included as a negative control in each experiment, and samples were then processed in batches of 10. PCR products were slot-blotted onto a nylon hybridization membrane (GeneScreen Plus, Du Pont, Boston) and hybridized to the appropriate ³²P-labeled internal oligonucleotide probe. After low stringency washes to retain any mismatched hybrids, filters were exposed to Kodak XAR5 film at –70°C with use of intensifying screens for up to 10 days.

DNA sequence analysis. Following electrophoresis, excised double-stranded PCR products were purified by GeneClean 11 (Bio 101, Inc., La Jolla, CA) treatment. The DNA was sequenced using [³⁵S]dATP (NEN, Boston) and the USB Sequenase 2.0 sequencing kit (U.S. Biochemical, Cleveland, OH) as described [16]. Samples were electrophoresed through 10% acrylamide/urea gels on BRL S2 gel systems, dried, and autoradiographed on Kodak XAR5 film.

Results

Histologic examination of the muscle biopsy specimens from the patients with CFS revealed nonspecific mild-to-moderate atrophy of type 2 fibers in ~40% of the samples. Mitochondria were slightly prominent on the Gomori trichrome stain. On ultrastructural examination, they were increased in number and size and showed mild-to-moderate pleomorphism in ~60% of the cases (figure 1).

A total of 32 (26.4%) of the 121 biopsy samples from patients with CFS were positive for enterovirus sequences by PCR, compared with 20 (19.8%) of the 101 from controls. A typical slot-blot result is shown in figure 2. Statistical analysis (χ^2 test) showed that the difference between these two groups was not significant.

Sequence analysis was carried out on the PCR products of six muscle biopsy specimens from patients with CFS to establish that exogenous virus was present. Sequence data read from autoradiographs were analyzed using the Sequence Analysis Software Package Version 7 (Genetics Computer Group, Madison, WI). No specific virus was revealed (se-

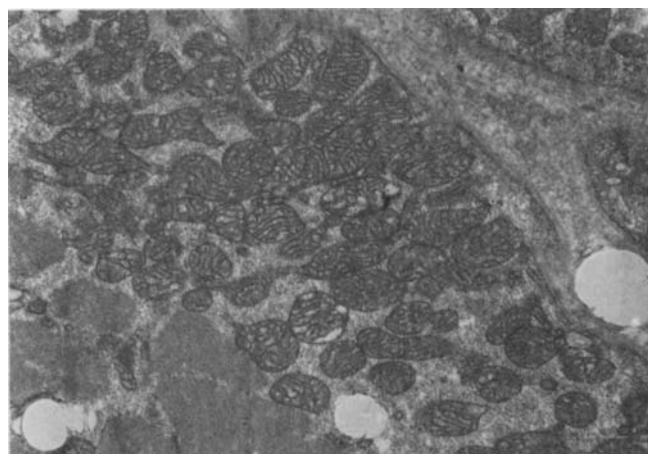


Figure 1. A subsarcolemmic aggregate of hyperplastic, pleomorphic mitochondria showing proliferation of the cristae (compartmentalization) in a specimen from a patient with CFS (magnification, $\times 23,000$).

sequence data are available for only about 20 of the 72 enterovirus types); however, the sequences detected were considerably different from our positive controls (coxsackievirus B3 and enterovirus 71) and therefore represented exogenous virus and not contamination. The PCR products sequenced also differed from each other, thus indicating that more than one enterovirus might be involved. In figure 3 the nucleotide sequences of coxsackievirus B3 and one of the PCR products from a patient with CFS are aligned and compared. It can be seen that there is a similarity of 78% between them, thereby indicating that the sequence amplified from the patient's specimen is of enterovirus origin.

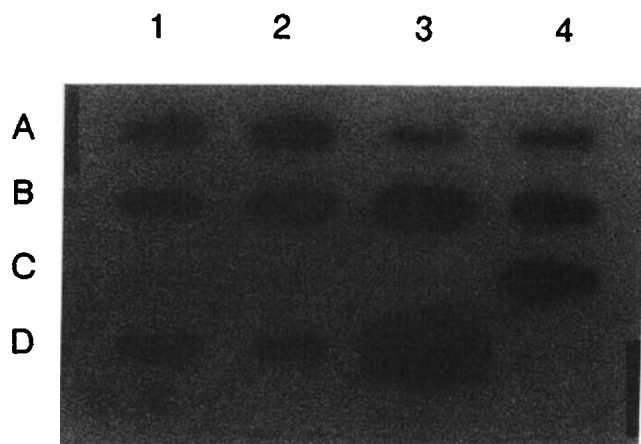


Figure 2. Detection of enterovirus amplification products in muscle biopsy specimens from patients with CFS by slot-blot assay and hybridization with internal ^{32}P -labeled oligonucleotide EP2. Lanes A1–A4 and B1–B3, samples from patients with CFS; lanes C1–C4 and D1–D2, samples from patients with other neuromuscular diseases; lane D3, positive control (a 1×10^3 dilution of CVB3-infected vero cell culture); and lane D4, negative control (uninfected MRC5 cell culture).

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CFS          . . . . . AATCGCTATCTCTAC . . . . . CGAAATACCCACACCC
CVB3 nt 451  CCGCCCTCGATCGGGCTATCTCTACTCGGGAGCACACCCCTCAAGCC 500
              AGTGGGCAGTATGTCGTATGGGCACTCATGCAGAGGAACCACTACT.
              || ||||| ||||| ||||| ||||| ||||| ||||| |||||
501  AGAGGGCAGTGTCTCTACGGGCACTC. TGCAGCCGAGACCACTACT 549

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Figure 3. Sequence data generated from a specimen from a patient with CFS that was aligned for comparison with the coxsackievirus B3 (positive control) sequence (CVB3). The example shown confirms that an exogenous viral sequence is present, with a similarity of 78% to the control virus.

Discussion

We have investigated a large group of patients with CFS for the presence of enterovirus in their muscle with use of the PCR. We found that 26.4% of the group of patients with CFS were positive, compared with 19.8% of the group of patients with other neuromuscular disorders, including dystrophies, neurogenic atrophy, and mitochondrial and metabolic problems. The difference is not significant, thus indicating that it is unlikely that enterovirus plays a major pathogenetic role in CFS. It may be, as has been postulated, that enteroviruses localize preferentially in damaged muscle [17]. Certainly there is evidence that muscle is affected in CFS; namely, electrophysiological membrane abnormalities [18] and impaired metabolic function on nuclear magnetic resonance studies [19] have been reported.

Ultrastructural analysis has revealed morphological abnormalities in muscle mitochondria (figure 1): hyperplasia, hypertrophy, pleomorphism, and a striking proliferation of the cristae giving rise to an appearance we have called *compartmentalization* [20]. These changes, which we found in 70% of a group of patients who were examined [20], are in keeping with an interference in mitochondrial respiration and the development of undue fatigue and myalgia on exertion. It has been suggested that, under certain conditions, defective mitochondria may have a selective advantage over normal wild-type organelles [21]. We postulate that, in some patients, a viral insult might therefore be followed by proliferation of mutant mitochondria and resultant impaired function.

Results of routine virological investigations of the epidemic cases have always been negative, thereby suggesting that an uncommon agent or variant may be involved [5]. Failure to detect an agent does not seem surprising in the sporadic cases that are not usually diagnosed until at least 6 months after the initial episode. Yousef and co-workers [12], however, reported chronic enterovirus excretion, together with serological evidence of circulating virus, in some cases, while molecular hybridization studies revealed enterovirus sequences in 20% of a group of 76 patients and none in control patients [13].

Neither we nor other investigators have found convincing evidence of necrosis or inflammation in muscle biopsy specimens from patients with CFS. This suggests that, if virus is present, it is at very low copy numbers, not enough to pro-

duce lytic infection or inflammation. Nonetheless, as shown by Oldstone [22] in studies of persistent viruses, such an infection may nevertheless produce severe impairment of function.

We have used the sensitive PCR in a search for enterovirus genome in muscle. Our first study revealed enterovirus sequences in 53% of the patients examined [11]. In the present study, we used the same primers but detected evidence of enterovirus in only 26% of patients. We used a different group of patients for comparison, i.e., ones with muscle damage due to other causes, and found that enterovirus could be detected in a similar number of them. Levels of enterovirus infection may vary in the community according to season and year, so that larger studies are critical. Our present findings are in accord with the hypothesis [17] that enterovirus may be found in various kinds of muscle damage.

To be sure that the sequences detected were indeed of exogenous virus, we have started sequence analysis. The PCR products from the six cases analyzed so far indicate that these are not contaminating laboratory viruses and may be of several different enteroviruses.

Our results do not exclude a role for persistent enteroviruses completely. Focal localization is a feature of some enterovirus infections [23], and limitations to the PCR may also restrict the number of enteroviruses detectable or preclude detection of the very low copy numbers that may be present. It is also possible that the virus has a "hit-and-run" effect. Other viruses could also be involved, e.g., herpesvirus type 6 [24]. Retrovirus has been reported in some cases [25], although we have not been able to reproduce those results [26]. The work on herpesvirus type 6 in patients with CFS suggests that reactivation of latent virus, probably due to selective immunodeficiency, may be a factor in the disease process [24].

Taking all the factors into consideration, our findings indicate that PCR studies on enteroviruses do not identify cases of CFS, and while the virus may play a role in disease, the illness is not dependent on persistent viral infection of muscle.

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