Amplification and identification of enteroviral sequences in the postviral fatigue syndrome

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Evidence from several sources has long suggested that enteroviruses might play a role in the postviral fatigue syndrome (PVFS). We used the most sensitive molecular virological method available at present, the polymerase chain reaction (PCR) amplification technique, to look for enteroviral copies in peripheral blood leucocytes and muscle from a well-defined group of patients. We demonstrated that our PCR method amplified a sequence common to a wide range of enteroviral serotypes. A highly significant number of the muscle biopsies (53%; $P = < 0.001$) from the patients were positive for enteroviral sequences. With regard to the leucocyte samples, 16% in both patient and control were positive. The PCR results on the peripheral blood leucocytes were in keeping with serological findings, in showing that the level of exposure to enteroviruses seemed to be the same in patients and controls: it was therefore of the greatest interest that patients were 6.7 times more likely to have enteroviral genome in their muscle. We conclude that persistent enteroviral infection plays a role in the pathogenesis of PVFS, also providing preliminary evidence that severe mitochondrial injury is one of the mechanisms involved.
Disorders which occur in epidemics suggest a common exposure to an infectious agent or toxin. The postviral fatigue syndrome (PVFS) was originally described in a series of epidemics occurring in the United States, Europe and Australia. The unusual nature of the illness, with its psychiatric features, led to the many problems in diagnosis which have been commented on in several reviews. On the basis of epidemiological studies, although no agent has been implicated, it could be concluded that an infectious agent was responsible: there appeared to be an incubation period of between 8 and 10 days, occasionally a little longer, and spread was by personal contact. An acute illness resulted from exposure and this lasted usually for a few weeks but occasionally persisted, with relapses, for months or years.

It seemed that a ubiquitous agent, possibly a virus, had to be the cause. The incubation period, length of clinical course and symptoms, predominantly involving muscle and nervous system, were all consistent with an enterovirus. These are known to circulate constantly in the population, producing not only large or small epidemics affecting previously unexposed individuals, especially in enclosed communities, but also single, sporadic cases. One of the epidemiological features of PVFS is that major epidemics are both preceded and followed by single cases in the same community, as was pointed out after both the first, major, Los Angeles epidemic and the Royal Free epidemic.

Each of the three enteroviral groups: Coxsackie, echo and polio have been pinpointed as causal agents at one time or another. The original reports strongly suggested polioviruses since the epidemics appeared at the same time as epidemics of poliomyelitis. One outbreak involved nurses working on a ward devoted to cases of poliomyelitis.

The findings in the 1948 Akureyri outbreak were particularly interesting. At first, the clinical features in the 400 affected children were thought to be those of polio. Overall, 5% of the male and 8% of the female population were affected, an attack rate calculated as 6.7%. The pattern of spread suggested an infectious agent but all attempts at isolation were negative, including analysis of faecal samples with intracerebral inoculation of monkeys and other laboratory animals. No increased serum titres to polioviruses were detected. Five years later, however, a large outbreak of poliomyelitis confirmed to be due to poliovirus 1, did occur in Iceland and it involved all areas of the country except where 'Akureyri disease' had occurred. In the rest of Iceland 50–95% of school children produced antibody to polio 1, but not the children in Akureyri. Later on, however, when immunisation to the virus
was given, in 1956, the latter children produced unusually high specific antibody titres, suggesting previous exposure. Another interesting feature was that when an American airman who had developed poliomyelitis in the 1955 Iceland epidemic, returned to Massachusetts, his homecoming was followed by a small, local outbreak of PVFS.

Epidemic cases in Adelaide, Australia, in 1949–51, again bore a strong clinical resemblance to poliomyelitis so that the investigations included inoculation of material from two cases, into monkeys. The animals showed a radiculitis of the sciatic nerves. These findings, however, have never been confirmed.

In contrast to all the occasions when no virus was isolated from the epidemic cases, echovirus type 9 (now classified as a Coxsackie virus) was grown from the faeces of patients in a small outbreak of PVFS in Lancashire which occurred just after a polio epidemic. The same virus was grown from the cerebrospinal fluid of one of the four typical cases described by Innes.

The strongest evidence, however, implicates Coxsackie viruses. These are known to be myotropic, as in Bornholm disease. They show an increased ability to replicate in muscle which has been denervated, or severely exercised. In the latter case, they resemble polioviruses which, in the days of the polio epidemics, were known to have a predilection to attack the motor neurones of recently active muscles.

In the 1980s, there were reports from Scotland that clusters of patients with PVFS had increased specific antibody titres to Coxsackie. Following this an enormous amount of serological work was carried out on cases of PVFS, including attempts to estimate specific IgM antibody. The results appeared at first to be promising with, in a very carefully categorised collection of cases, 40% having increased titres of specific antibodies. When, however, a large, well-controlled epidemiological study was done, no difference in antibody titres was detectable between cases and controls. Similarly, in our latest investigation, in which we looked for persistent virus in muscle samples using the polymerase chain reaction, we found that specific Coxsackie antibody titres were almost identical in the patients and controls. The serological state thus appears to reflect the circulation of these viruses in the community and bears no relationship to whether or not the virus is causing muscle problems in the individual.

Nonetheless, at the time, the serological data led investigators to try to identify enteroviral genome in muscle, using molecular hybridisation techniques. This work provided the first evidence that enteroviruses were persistent in patients' muscle, with 20%
of cases having positive results.\textsuperscript{22} We then decided to apply the polymerase chain amplification technique to a well-defined group of patients with PVFS, comparing the results to a control group. This technique is the most sensitive molecular virological investigation at the moment, making possible the detection of very low copy numbers of virus.

CASE SELECTION

In any investigation of PVFS, the selection of cases is a critical factor. We applied the following criteria which we have discussed previously,\textsuperscript{1,21} together with the working case definition outlined by Holmes and colleagues at the National Institutes of Health.\textsuperscript{23} All the 60 cases studied reported that their illness started with a febrile illness accompanied by respiratory or gastro-intestinal symptoms, followed by severe, unremitting fatigue and myalgia which lasted for more than one year. The fatigue was made much worse by exercise. Most of the patients also complained of poor concentration and a falling-off in intellectual performance. One-third had had episodes of tachycardia. The 60 patients comprised 27 males (mean age 34.6 years) and 33 females (mean age 39.6 years), all of whom considered themselves to have been in good physical health prior to their illness. Rigorous investigations, as outlined,\textsuperscript{23} and reported in detail\textsuperscript{21} revealed no other cause for the fatigue.

CONTROL GROUP

It was not considered justifiable to biopsy normal individuals and therefore the control muscle samples were taken from patients admitted for routine surgical procedures. A 1.5 mm\textsuperscript{3} of skeletal muscle, was obtained from the operative site. The cases were age and sex-matched to the patients with PVFS as far as possible; they were all from the same catchment area and were in the hospital at the same time. The group comprised 28 females (mean age 48.2 years) and 13 males (mean age 56 years), none of whom had any evidence of muscle problems or complained of fatigue. Their clinical diagnoses were: benign breast lump (in two cases); carcinoma of breast (15); vascular surgery (10); partial gastrectomy (2); partial colectomy (4); thyroid lobectomy (3); repair of abdominal hernia (2); cholecystectomy (2) and pinning of fractured hip (1).
MUSCLE BIOPSIES

The patients with PVFS had a needle muscle biopsy carried out under local anaesthetic. For convenience, the three cores of skeletal muscle were obtained from the right vastus lateralis, using the modified UCH needle. The core which was destined for PCR analysis was removed from the cannula using an aseptic technique, placed in a sterile container, snap-frozen and stored in liquid nitrogen until required. A further one and a half cores were snap-frozen for subsequent histological examination and the remaining half core was fixed in 2% gluteraldehyde for ultrastructural analysis.

SETTING UP THE POLYMERASE CHAIN REACTION (PCR) AMPLIFICATION TECHNIQUE

It was first necessary to devise a protocol which would enable us to detect as wide a range as possible of the different enteroviruses. There are more than 70 serotypes of enterovirus, of which only 10 have had their nucleic acid sequences fully determined. Previous studies, however, have shown that there is approximately 80% homology between the different serotypes in the 5' non-translated region. Therefore this was the site selected for amplification; the primer oligonucleotides for the polymerase chain reaction and the internal probe oligonucleotides, were selected from this region. The enteroviral primers gave rise to a DNA band of 414bp.

As the enteroviruses (members of the Picornaviridae) exist as RNA genomes, it is not possible to carry out direct PCR amplification. It is necessary to synthesise cDNA from the RNA (first strand synthesis is sufficient) and then amplify from the cDNA. To test the validity of the PCR protocol a number of different enteroviral types were grown in Vero or Hep2 cells. RNA was isolated and cDNA-PCR performed. Reaction conditions were optimised by titration of magnesium chloride concentrations and by careful choice of annealing temperature. The automatic thermal cycler was set to give 35 cycles of 1 minute at 94 °C, 1 minute at 55 °C and 1.5 minutes at 72 °C.

As a control for the quality of the RNA obtained and for the enteroviral amplification reaction (especially in the case of negative results), we included amplification of a 'house-keeping' gene. We used Ableson (abl) tyrosine kinase gene primers since they give
rise to a positive product, a band of 218bp, in all human RNA samples. Only those samples which gave rise to a positive abl band were used in the enteroviral PCR analysis.

The oligonucleotide PCR primer and probe sequences were as follows:

- **ABL1**: 5' CAG CGG CCA GTA GCA TCT GAC TT 3'
- **ABL2**: 5' TGT GAT TAT AGT AGA CCC GGA G 3'
- **EP1**: 5' CGG TAC CTT TGT GCG CCT GT 3' (bp 64 to 83)
- **EP2**: 5' TAT TGA GCT AGT TGG TAG TCC TCC GG 3' (bp 430 to 455, internal probe)
- **EP4**: 5' TTA GGA TTA GCC GCA TTC AG 3' (bp 459 to 478)

The high sensitivity of the PCR technique may lead to false positive results if a sample is contaminated by minute amounts of target sequence molecules which are then amplified. Therefore, we used dedicated pipettes and samples were processed in batches of ten only. Negative experimental controls included calf thymus DNA, normal human muscle RNA, Vero/Hep2 cell RNA and reaction tubes containing PCR reaction mix but no nucleic acid.

The PCR products were examined by agarose gel electrophoresis, ethidium bromide staining and ultraviolet light visualisation. This was followed by southern blot analysis and hybridisation with an internal probe end-labelled with either digoxigenin-dUTP or [32P]-ATP. Alternatively, PCR products were slot-blotted directly onto a hybridisation membrane and hybridised with the same probe. Hybridisation filters were processed for the digoxigenin colour reaction, or the blots were exposed to Kodak XAR5 film at -70 °C with intensifying screens.

Table 1 shows a list of the results obtained with the control enteroviruses. These results confirmed that a range of enteroviral types, including Coxsackie A and B, poliovirus, echovirus and higher enteroviruses could be detected using the 5' NTR PCR primers and probe. They also show that the primers did not give rise to false positive product bands in normal human muscle, uninfected host cells or measles virus-infected cells.

The sensitivity of the cDNA-PCR technique was assessed by amplifying RNA from CBV3-infected cells serially diluted with RNA from uninfected cells. Dilutions ranged from 1 in 10 to 1 in 10^7 and a positive signal was visible under ultraviolet light at the
Table 1  A list of the results obtained with the control enteroviruses

<table>
<thead>
<tr>
<th>Virus</th>
<th>Host Cell</th>
<th>PCR</th>
<th>EP2 Internal Probe</th>
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<tbody>
<tr>
<td>Coxsackie</td>
<td>A16</td>
<td>MRC5</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>A21</td>
<td>MRC5</td>
<td>+</td>
</tr>
<tr>
<td>Coxsackie</td>
<td>B1</td>
<td>Vero</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>B2</td>
<td>Vero</td>
<td>+</td>
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<tr>
<td></td>
<td>B3</td>
<td>Vero</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>B4</td>
<td>Vero</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>B5</td>
<td>Vero</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>B6</td>
<td>Vero</td>
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</tr>
<tr>
<td>Poliovirus</td>
<td>P1</td>
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<tr>
<td></td>
<td>P2</td>
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<td>+</td>
</tr>
<tr>
<td></td>
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<td>+</td>
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<td></td>
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<td>-</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>Vero</td>
<td>+</td>
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<tr>
<td>Enterovirus</td>
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<td>+</td>
</tr>
<tr>
<td></td>
<td>70</td>
<td>MRC5</td>
<td>+</td>
</tr>
<tr>
<td>Controls</td>
<td>Measles virus</td>
<td>MRC5</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>MRC 5 (human lung)</td>
<td></td>
<td>-</td>
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<tr>
<td></td>
<td>HEp (human hepatoma)</td>
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<td>-</td>
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<tr>
<td></td>
<td>Vero (monkey kidney)</td>
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<td>-</td>
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<tr>
<td></td>
<td>Muscle biopsy (human normal)</td>
<td></td>
<td>-</td>
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* = weak positive signal

Compilation of results obtained with a range of enteroviral types PCR amplified with enteroviral specific oligonucleotide primers. The internal probe—oligo EP2, hybridizes to varying degrees of the different enteroviral types DNA. This highlights the extent of sequence variability between enteroviral types. (Note Echovirus 22 gave a negative result—other groups have reported similar findings and it is now thought that Echo 22 has been wrongly classified)

1 in 10^4 dilution. When the products were subsequently probed with a labelled internal oligonucleotide, it was possible to detect a signal at the 1 in 10^6 dilution. A 1 in 10^3 dilution of CBV3-infected/uninfected cells was used as the positive control in each human experiment.

We concluded that, although there is sequence variation within the enteroviral group, there is sufficient conservation to regard the PCR primers we selected from published nucleotide sequences as being of broad specificity for a wide range of enteroviruses.

RESULTS

No significant abnormalities were found in the routine laboratory tests, including no increase in serum creatine kinase concentrations.
The patients and controls were studied at the same time period and were from the same catchment area. Their serological results were almost identical: 25% of patients and 24.1% of controls had increased antibody titres to Coxsackie viruses, Epstein-Barr or cytomegalovirus. This indicated that the two groups had had a similar exposure to viruses in the community.

Histological examination of the muscle biopsies revealed only a focal, mild or moderate, non-specific atrophy of type 2 fibres in 50 of the 60 patient samples. There was no evidence of inflammation, necrosis or regeneration. On histochemical stains, however, mild mitochondrial prominence was detected while ultrastructural examination revealed obvious evidence of mitochondrial damage in 45 of the cases (Fig. 1). The findings consisted of swelling, pleomorphism, proliferation of the cristae, with the apparent formation of compartments and focal vacuolation. Clusters of these injured organelles were visible beneath the sarcolemmal membrane. Occasional autophagolysosomes were present and there was a mild excess of both glycogen granules and lipid. A detailed description of these changes will be given elsewhere.

PCR amplification, followed by hybridisation with an internal probe, gave rise to a positive signal in 32 of the 60 patients in the study, and no signal in 28 (53.3% positive). In contrast, of the 41 control cases, 6 were positive and 35 negative (15% positive). The 6 positive cases all had neoplasia—5 carcinoma of breast and 1 carcinoma of colon.

Application of the χ² test showed a highly significant difference between the patient and control groups. The relative risk of a PVFS patient having an enteroviral infection was 6.7 \((35 \times 32/6 \times 28)\) as an odds ratio with a confidence interval of 2.4 to 18.2 \((χ²\) with correction for continuity = 13.94 when \(α = 0.05\), df = 1, therefore \(P = <0.001\)).

Of the patients with PVFS, enteroviral sequences were detected in 12 of the 27 males and 20 of the 33 females. There was no correlation between duration of disease and PCR result. Nor was there any correlation between PCR result and presenting symptoms, i.e. acute upper respiratory tract, gastrointestinal, flu-like or infectious mononucleosis-like infection. Three patients related the onset of their disease to an episode of herpes zoster or varicella: of these, one was negative and two positive.

Autoradiographs were exposed for up to ten days but most positive signals were detected after a 16 hour exposure. It must be stressed however, that the strength of hybridisation signals...
Fig. 1 Ultrastructural examination of skeletal muscle biopsy from a patient with the postviral fatigue syndrome: pleomorphic mitochondria showing proliferation of cristae with apparent formation of compartments. Numerous glycogen granules are also present (× 39 000).
from the patients' samples was much weaker than that of the CBV3-infected control cells. This indicates a very low number of viral copies: our estimate is one virus particle per 2000 cells.

DISCUSSION

We have investigated a well-defined group of 60 patients with PVFS for the presence of persistent enteroviruses in their muscle. We found enteroviral genome in the tissues of 53% of patients, as compared to 15% of control cases, a highly significant finding.

Serological studies done at the same time indicated that both patients and control subjects had an almost identical exposure to community viruses. It is therefore of the greatest importance that patients with PVFS were 6.7 times more likely to have enteroviral persistence in their muscle.

At the time of the study it was not considered ethically justifiable to biopsy normal individuals and we therefore turned to a group of patients, known to have no muscle problems and no complaints of undue fatigue, who were undergoing surgery for a variety of different conditions. These included removal of benign breast lumps, vascular surgery and resection for colonic or breast carcinoma. None of the patients with benign breast lumps or vascular problems proved to have persistent enteroviruses but 5 of the 12 cases of breast carcinoma and 1 of the 3 cases of colonic carcinoma were positive. It is tempting to suggest that the neoplasia, which is known to be associated with profound defects in immunity, might be an important causal factor in the viral persistence. We are now planning to encourage healthy relatives of patients with PVFS to undergo the simple, minor needle muscle biopsy procedure.

With regard to the 47% of the patients with PVFS in whom enteroviral genome was not detectable, several explanations may be given: the viral infection may be focal (as is known to occur in Coxsackie myocarditis\(^ {28} \)) and the lesion site could therefore have been missed on biopsy. The PCR might not be sensitive enough to detect very low copy numbers of virus, or the internal probe could be too type-specific. We hope to overcome this latter problem by using nested primers.

We now have preliminary PCR data which suggests that a retrovirus or endogenous retroviral sequences are involved in PVFS. We have amplified HTLV II \textit{gag} \textit{sequences from DNA and HTLV I/II pol sequences from DNA and RNA isolated from
the peripheral blood buffy coat cells of 15 patients but not from 15 controls. Primers for HTLV I/II env and tax regions did not give rise to any amplification products (unpublished results). These findings are undergoing further investigation.

As stated earlier, the strongest evidence supports enteroviral infection of muscle in the aetiology of PVFS, but other viruses have been implicated. The Epstein-Barr virus is known to cause a somewhat similar illness, the chronic mononucleosis syndrome, while three of our patients related the onset of their disorder to varicella-zoster infection. Thus it is essential to search for these and other agents in the muscle specimens. There is also the possibility that the enteroviruses might simply be opportunistic viruses persisting in a subtly changed cellular environment (see above).

The PCR technique does not enable us to determine which cell is host to the infecting enterovirus. Since histological examination reveals no evidence of any inflammatory infiltrate, it is likely to be muscle cells but this remains to be determined. Because of the difficulties in setting up an in situ hybridisation technique in the biopsies, we first carried out a parallel PCR study on the peripheral blood leucocytes (obtained from buffy coat samples) of 20 patients and 20 normal individuals, to determine whether enterovirus could be detected. The RNA was prepared and amplified in a similar manner to that from muscle samples. The results showed no difference between the two groups (16% positivity in each—unpublished results).

Enteroviruses have now been demonstrated by in situ techniques, in both animal models of myocarditis and myositis and, very recently, in human myocarditis. These results have illustrated one of the problems which may occur when the virus is persistent, namely the focal nature of the lesions. As regards PVFS, we do not know if the infection is focal or not but we do know, based on the results of our PCR study, that there is likely to be a minute amount of virus present—1 viral genome per approximately 2000 cells. Replication of the persisting viruses may also be defective. The usual ratio of positive (coding) strand viral RNA to negative copy for infectious virus is 100:1 but in the case of a persistent virus, it has been reported that approximately equal amounts of positive and negative strands are present.

The very low level of enterovirus found in the muscle of patients with PVFS, is in keeping with the complete absence of cell necrosis or inflammation. It does, however, make it difficult to explain the
overwhelming fatigue of which the patients complain. For this reason, our finding of mitochondrial damage may be important. Oldstone et al.\textsuperscript{32} have shown that persistent viruses may completely prevent the specialised functions of the host cell, while apparently producing no other adverse effects, including necrosis. The mitochondrial pleomorphism with proliferation of cristae may indeed be a response to interference with the energy metabolism required for normal skeletal muscle activity.

Although our results draw attention to a peripheral cause for the fatigue in PVFS, certain clinical features suggest that there is also central nervous system involvement. Whether this is due to local infection, or to a systemic effect produced by cytokines whose release is triggered by virus,\textsuperscript{33} cannot be determined at present. Since there are now several mouse models of enteroviral persistence, it will be possible to investigate this area in greater detail in future.

Finally, no one has yet demonstrated whether or not full-length enterovirus or complete virion is present in cases of PFS, nor do we know if particular serotypes are involved. Whether it is defective virus, an ineffective immune response or host genetic factors which plays the principal role in aetiology, remain unanswered questions.

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