Search for picornaviruses at onset of inflammatory myopathy

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Abstract
Picornaviruses may not play a role as persistent agents in the inflammatory myopathies, but it is still thought likely that they may act as triggers of an autoimmune process. Forty one muscle biopsy specimens, taken from three weeks to six months (mean four months) after onset, were examined using three different picornaviral primers and PCR. Moderate to severe disease activity was evident in all specimens. The results were compared with those of 18 biopsy specimens examined later in the disease course, and with specimens from 27 patients with non-inflammatory myopathies. All results were negative. Thus, even as early as three weeks after clinical disease appears, picornaviruses are not detectable in these disorders.

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Keywords: picornaviruses, inflammatory myopathy, PCR.

Polymyositis, dermatomyositis and inclusion body myositis are idiopathic inflammatory myopathies with well defined clinical, immunological and pathological features, but an unknown aetiology. There is a primary attack on myocytes by cytotoxic T lymphocytes and macrophages, but the surface antigen(s) to which these cells are directed is unknown. Various viruses, especially myotropic enterovi- ruses, have been put forward as candidates. All attempts to isolate these agents from typical patients have failed, but the original molecular hybridisation studies were positive.1 2

Since then, however, investigators using highly sensitive and specific PCR, and carrying out further in situ studies have failed to detect candidate viruses including coxsackie, mumps, encephalomyocarditis (EMC), adenovirus, human lymphotrophic virus types I and II, and HIV. 3 4 It has been concluded, therefore, that persistent coxsackie or related enterovirus infection is unlikely to be the continuing stimulus for disease.

An autoimmune origin for idiopathic inflammatory myopathies is supported by a wide range of evidence, however, and viruses could play a role in triggering the autoimmune attack, presumably before being eliminated. 5 It is therefore of the utmost importance to look for the agent early in the disease process; this has not been done previously. In most of the studies cited, the time of biopsy in relation to disease is not given but where it is, the mean has been from 45 to 78 months. 6 7 8

We have examined 41 muscle biopsy specimens taken at presentation in patients with an idiopathic inflammatory myopathy. The earliest cases were seen at three weeks after the first symptoms and the latest at six months (mean four months). We compared the findings with those in 18 patients seen later in their disease course (mean 13 months) and in 27 patients with non-inflammatory myopathy.

Methods

PATIENTS
Forty one patients (19 men and 22 women), aged from 15 to 81 years, with an acute or subacute onset of muscle weakness with or without a skin rash, underwent biopsy of the vastus lateralis muscle. They were hospitalised,

Table 1  Clinical details of patients with inflammatory myopathy

<table>
<thead>
<tr>
<th>Clinical subgroup</th>
<th>n</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Disease duration (mean)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biopsy specimens taken at onset</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PM</td>
<td>27</td>
<td>23–74</td>
<td>15 M, 12 F</td>
<td>3–6 months (4)</td>
</tr>
<tr>
<td>DM</td>
<td>6</td>
<td>19–67</td>
<td>2 M, 4 F</td>
<td>6 weeks to 6 months (2.5 months)</td>
</tr>
<tr>
<td>PM or DM with neoplasia</td>
<td>4</td>
<td>46–70</td>
<td>4 F</td>
<td>3–5 months (4)</td>
</tr>
<tr>
<td>Juvenile DM</td>
<td>2</td>
<td>15, 17</td>
<td>1 M, 1 F</td>
<td>3 weeks (2)</td>
</tr>
<tr>
<td>IBM</td>
<td>2</td>
<td>67, 81</td>
<td>1 M, 1 F</td>
<td>5 months, 6 months (5.5 months)</td>
</tr>
<tr>
<td>Total</td>
<td>41</td>
<td>15–81</td>
<td>19 M, 22 F</td>
<td>3 weeks to 6 months (4 months)</td>
</tr>
<tr>
<td>Biopsy specimens taken later in disease course</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PM</td>
<td>5</td>
<td>41–69</td>
<td>2 M, 2 F</td>
<td>8 months to 3 years (12 months)</td>
</tr>
<tr>
<td>PM or DM with connective tissue disease</td>
<td>13</td>
<td>26–78</td>
<td>10 M, 3 F</td>
<td>10 months to 3 years (15 months)</td>
</tr>
<tr>
<td>Total</td>
<td>18</td>
<td>26–78</td>
<td>13 M, 5 F</td>
<td>8 months to 3 years (13 months)</td>
</tr>
</tbody>
</table>

PM = polymyositis; DM = dermatomyositis; IBM = inclusion body myositis; n = number of cases.
Table 2  Oligonucleotide PCR primer and probe sequences

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence ($)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Entoviral primers¹</td>
<td>EP1:5' CGG TAC CTT TGT GCG CCT GT 3'</td>
</tr>
<tr>
<td>Entoviral primers²</td>
<td>EP4:5' TTA GGA TTA GCC GCA AG 3'</td>
</tr>
<tr>
<td>Entoviral primers³</td>
<td>EP2:5' TAT TGA GCT AGT TGG TAG TCC GTT 3' (internal probe)</td>
</tr>
<tr>
<td>Theiler's virus primers</td>
<td>Z1: 5' CAA GCA CTT CTG TTT CCC CGG 3'</td>
</tr>
<tr>
<td>Theiler's virus primers</td>
<td>Z3: 5' ATT GTC ACC ATA AGC AGC CA 3'</td>
</tr>
<tr>
<td>Theiler's virus primers</td>
<td>TV1: 5' GTG ACA CTT GGA CTA GAG GT 3'</td>
</tr>
<tr>
<td>Theiler's virus primers</td>
<td>TV2: 5' ACT CTC TGG TGA TGA TGG TA 3'</td>
</tr>
<tr>
<td>Theiler's virus primers</td>
<td>ABL1: 5' CAG CGG CCA GTC GCA TCT GAC TT 3'</td>
</tr>
<tr>
<td>Theiler's virus primers</td>
<td>ABL2: 5' TGT GAT TAT AGC CTA AGA CCC GGA G 3'</td>
</tr>
</tbody>
</table>

extensively investigated, serum creatine kinase activities were established, and electromyography performed. A further 18 well characterised patients in whom the disease has been present longer also underwent biopsy. The clinical details are shown in table 1.

CONTROLs
The control group comprised 27 patients (16 men), aged 26–75 years, with a variety of other non-inflammatory neuromuscular disorders, who had undergone diagnostic muscle biopsy. Their diagnoses included alcoholic and mitochondrial myopathy, muscular dystrophy, motor neurone disease, amyloidosis, and sarcoidosis.

MUSCLE BIOPSY SPECIMENS
Three cores of skeletal muscle were obtained from each patient using a standard technique.³ A fresh tissue cube, 20 mm³, was snap frozen in liquid nitrogen pending PCR. The remainder of the muscle was orientated to form a cylinder 25 mm³ in cross-section and 6 mm long, from which approximately 70–80 sections, of up to 1000 fibres, could be stained.

PCR AND ELECTROPHORESIS
RNA was prepared from the 20 mm³ tissue cube and three PCR reactions were performed, each on 1 µg nucleic acid extracted from each specimen, as described previously.⁴ To ensure that the samples were of amenable quality, primers for the ubiquitous human gene Abelson tyrosine kinase (ABL), which spans an intron–exon junction and can therefore only be amplified from RNA/cDNA, were used.⁴ Great care was taken to avoid contamination by RNA during amplification: negative and B3 infected positive controls were included.

PCR PRIMERS

Specificity
Two pairs of previously described entero virus primers¹⁴ and one pair of Theiler’s murine encephalomyelitis virus (TMEV) primers, based on the TMEV sequence in the GCG Database, were used (table 2). Primers EP1 and EP4 amplify a common sequence from the 5' end of the polyivirus, echovirus and higher enterovirus types,¹⁵ as do Z1 and Z2 primers which detect 60 of 66 EV types tested.¹⁶ The TMEV probe hybridises to human enteroviruses and EMC sequences.¹⁵

Sensitivity
To assess sensitivity, known quantities of RNA from coxsackie B3 infected Hep2 cells (200 virus genomes per cell) were diluted serially with RNA from uninfected cells. The infected cells had been washed several times with phosphate buffered saline during harvest to remove any free virus particles from the culture medium. Dilutions from 1 in 10 to 1 in 10³ were made. A positive band was visible by ethidium bromide staining at the 1 in 10⁴ dilution, and after hybridisation with an internal probe a positive signal was visible in autoradiographs for dilutions down to 1 in 10⁶. The copy number of viral particles which could be detected was calculated by densitometric comparison with known amounts of a CBV3 cDNA construct. It was estimated that 20 viral genomes could be detected on PCR by this method. RNA at a dilution of 1 in 10⁴ from coxsackie B3 infected/uninfected cells was used routinely as the positive control in each experiment.

SOUTHERN BLOT HYBRIDISATION
To confirm that any positive PCR products were of viral origin, the amplified PCR products were Southern blotted onto nylon membranes (Gene-Screen Plus) and probed with an internal oligonucleotide probe.⁶

Results
The cases were classified according to standard criteria¹ and represented all subgroups (table 1). The degree of disease activity was assessed, based on fibre necrosis, mononuclear cell infiltration, fibre regeneration, and endomyosial fibrosis.³ The 41 specimens taken at onset showed moderate to severe disease activity. Mild to moderate features were present in the 18 later specimens. The control group diagnoses were confirmed histologically.

No amplification bands of the correct size were detected in any of the patient or control samples. Occasional non-specific fragments, shown by DNA sequence analysis not to be of viral origin, were found in some of the PCR products using the EP1/EP4 primers. All specimens were positive for the 218 base pair fragment of the ABL gene, indicating that sufficient good quality RNA was present. Coxsackie virus RNA was easily detected in the positive control using the three picornaviral primers.

Discussion
No picornaviral RNA sequences were detected in muscle from three weeks to six months after onset in patients with polymyositis, dermatomyositis and inclusion body myositis. Biopsy evidence of moderate to severe disease activity was present in all patients. The mean time of examination for the group was four months, and, in two cases of juvenile dermatomyositis,
the onset was only three weeks previously and in
another six (four with dermatomyositis and
two with polymyositis) it was six weeks. Other
authors have reported similar negative find-
ings, but have not stated the time of biopsy or
have taken it very late in the disease course—
that is, from 18 to 34 years. The 18 biopsy
specimens tested later in the course of the dis-
ease (table 1), at a mean of one year, were also
negative. These patients were on steroids while
the first group were not. A control group of
patients with a wide range of non-
inflammatory muscle disorders was also nega-
tive. Three different enteroviral primers were
used, the RNA was established to be of amplifi-
able quality and the control samples gave the
expected positive and negative results.

Could we have missed detectable picornavi-
ruses? This seems unlikely because the PCR
sequences in the two enteroviral primers used
were selected to amplify conserved sections of
the viral genome and are known to detect a
wide range of enteroviruses,2 4 while the
TMEV probe hybridises to EMC sequences and
human enteroviruses.3 The latter was selected
because in situ localisation of TMEV had been
reported in three of five patients with
dermatomyositis.1 7 The sensitivity of our PCR
assay was calculated as the equivalent of
detecting 20 molecules of viral nucleic acid in
a muscle sample, more sensitive or similar to
that in other studies.2 7 4 Our sample size, 20 mm2,
was also comparable.2 7 4

The original studies looking for enter-
oviruses in the inflammatory myopathies,
including our own preliminary one, were posi-
tive.1 7 8 In a large study of patients with
chronic fatigue syndrome we also reported
positive results.9 It has become apparent, how-
ever, that the methods used at the time by our-
selves and others were not as specific as was
hoped (see below). In the study reported here,
the PCR products were subjected to Southern
blot analysis before hybridisation with the virus
specific probe, as in recent, similar studies.2 7 4
Using this technique, any non-viral
PCR products which appear can be elimi-
nated.5 7

The evidence from the previous hybridisa-
tion studies has been described as “both confus-
ing and conflicting”10 the reasons for
false positive results discussed in detail.2 4 7
However, there has been a recent report that
enteroviral RNA is detectable in approximately
25% of patients with inflammatory myopathy
or chronic fatigue syndrome4 and obviously
these findings have to be taken into considera-
tion.

The whole question of the persistence of
these agents in human disease has been
debated recently, with the arguments for and
against ably marshalled.2 4 In his authoritative
chapter, Dalakas reviews this same question.7
He also points out that, as far as is known,
there is no receptor for enteroviruses on human
muscle fibres. His conclusion has to be
accepted, namely that “it is unlikely but not
impossible that persistent enteroviral infection
plays a role in the pathogenesis of the
inflammatory myopathies”.7

Studies on animals confirm the difficulty of
demonstrating a role for enteroviral persistence
even when myositis is initiated by a coxsackie
virus infection. A recent report indicates that
3–12% of muscles tested one year after inoc-
ulation were positive for enteroviral RNA. The
PCR products, however, were not subjected to
Southern blot analysis and there was no corre-
lation with clinical or histological features.10
A role for these organisms in initiating an
autoimmune myopathic process, however, is
still widely accepted.1 In this study we there-
fore concentrated our search on tissue taken as
early as possible in the disease course. Our
negative findings, although important, do not
completely rule out picornaviruses in the aeti-
ology of the inflammatory myopathies as it may
be that clinical onset of disease in humans
occurs after viral elimination.

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