Short reports

Search for picornaviruses at onset of inflammatory myopathy

W M H Behan, JW Gow, K Simpson, P O Behan

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.
(*J Clin Pathol* 1996;49:592-594)

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.¹ 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 enteroviruses, have been put forward as candidates.¹ 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 lymphotropic virus types I and II, and HIV.¹⁻⁴ 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.¹ 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.¹⁻⁴

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

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

PM = polymyositis; DM = dermatomyositis; IBM = inclusion body myositis; n = number of cases.

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Accepted for publication 21 March 1996

J Clin Pathol: first published as 10.1136/jcp.49.7.592 on 1 July 1996. Downloaded from http://jcp.bmj.com/ on April 26, 2023 by guest. Protected by copyright.

Table 2 Oligonucleotide PCR primer and probe sequences

Enteroviral primers ⁵	EP1:5' CGG TAC CTT TGT GCG CCT GT 3' EP4:5' TTA GGA TTA GCC GCA TTC AG 3' EP2:5' TAT TGA GCT AGT TGG TAG TCC TCC GG 3' (internal probe)
Enteroviral primers ⁶	Z1: 5' CAA GCA CTT CTG TTT CCC CGG 3' Z3: 5' ATT GTC ACC ATA AGC AGC CA 3'
Theiler's virus primers	TV1: 5' GTG ACA CTG TTG GCA TGC GT 3' TV2: 5' ACT CTC TGG TGA TGA TGG TA 3'
Abelson control gene primers ⁵	ABL1: 5' CAG CGG CCA GTA GCA TCT GAC TT 3' ABL2: 5' TGT GAT TAT AGC CTA AGA CCC GGA G 3'

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.⁵ 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 mm² 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.⁵ To ensure that the samples were of amplifiable 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.⁵ 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 enterovirus primers⁵ 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 poliovirus, echovirus and higher enterovirus types,⁵ as do Z1 and Z2 primers which detect 60 of 66 EV types tested.⁶ The TMEV probe hybridises to human enteroviruses and EMC sequences.³

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 10⁷ 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 10³ 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.⁵

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 endomysial 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 findings, 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.²⁻⁴ The 18 biopsy specimens tested later in the course of the disease (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 negative. Three different enteroviral primers were used, the RNA was established to be of amplifiable quality and the control samples gave the expected positive and negative results.

Could we have missed detectable picornaviruses? 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,⁵ 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 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.²⁻⁴ Our sample size, 20 mm³, was also comparable.²⁻⁴

The original studies looking for enteroviruses in the inflammatory myopathies, including our own preliminary one, were positive.^{1,2,7,8} In a large study of patients with chronic fatigue syndrome we also reported positive results.⁹ It has become apparent, however, that the methods used at the time by ourselves 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.^{2,3,7} Using this technique, any non-viral PCR products which appear can be eliminated.^{2,7}

The evidence from the previous hybridisation studies has been described as “both confusing and conflicting”⁴ and the reasons for false positive results discussed in detail.²⁻⁴ However, there has been a recent report that enteroviral RNA is detectable in approximately 25% of patients with inflammatory myopathy or chronic fatigue syndrome⁸ and obviously these findings have to be taken into consideration.

The whole question of the persistence of these agents in human disease has been

debated recently, with the arguments for and against ably marshalled.^{2,8} 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 inoculation were positive for enteroviral RNA. The PCR products, however, were not subjected to Southern blot analysis and there was no correlation 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 therefore 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 aetiology of the inflammatory myopathies as it may be that clinical onset of disease in humans occurs after viral elimination.

This work was kindly supported by the Barclay Trust of Glasgow University. The Persistent Virus Disease Foundation, McFeat Bequest, and Institute of Neurological Sciences Research Trust provided additional help.

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