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FORUM



There is no Evidence for Persistent Enterovirus Infections in Chronic Medical Conditions in Humans

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INTRODUCTION

Enteroviruses, together with the rhinoviruses, aphthoviruses, and the cardioviruses, belong to the family of *Picornaviridae*.¹ The genus enterovirus can be further divided into polio-, ECHO-, Coxsackie A and B-, and the enteroviruses 68 to 71.

Enteroviruses are the prototype of positive-strand RNA viruses since the genome is similar to eukaryotic mRNA. A small virally encoded protein, Vpg, is covalently attached to the 5' end and a poly(A) tail is present at the 3' end of the viral RNA. Once the virus has entered the host cell, uncoating is completed and the viral RNA is released into the cytoplasm of the cell where the RNA is directly translated into a polyprotein¹ of M_r about 200 000. Directly after infection, host cell protein synthesis is shut-off. Evidence for the mechanism of poliovirus shut-off points towards the inactivation of initiation complex eIF-4F, in which the cleavage of a cellular protein p220 by the 2A protease is thought to be involved.²

Virus progeny are released by cell-lysis which is induced by the infection itself. The virus can subsequently spread throughout the body until neutralising antibodies arise that stop further spreading and eliminate the virus. At that point, virus cultures will become negative. The role of cellular immunity seems to be of less importance than that of antibody. This is consistent with an obligatory cell-lysis forming part of the *in vivo* infectious cycle. Thus, chronic enterovirus infections are only known to occur in patients with agammaglobulinaemia³ or neonates, in whom the immune system is still immature.⁴ It is possible to isolate virus from such patients, even in the chronic stage of the infection. In normal health, enteroviral infection may follow a biphasic course, with a secondary viraemia with dissemination of the infection to target organs such as heart, brain, pancreas, or muscle.⁵

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Abbreviations used: DM, dermatomyositis; IBM, inclusion body myositis; IDC, idiopathic dilated cardiomyopathy; IDDM, idiopathic diabetes mellitus; ISH, *in situ* hybridisation; PM, polymyositis; PPS, post-polio syndrome; PVFS, post-viral fatigue syndrome.

Chronic inflammatory diseases such as polymyositis and chronic myocarditis are also associated with enteroviral infections. These diseases are regarded as autoimmune diseases, possibly induced by a preceding enteroviral infection. In the past decade, however, several papers have been published which describe the detection of enteroviral RNA in skeletal and heart muscle of such patients. These findings suggest that the virus persists after the acute phase of infection and that the virus itself may actually predispose to the maintenance of disease. These diseases, however, have in common that the virus itself can not be isolated anymore. Persistence of enterovirus has also been claimed for several other diseases as the post viral fatigue syndrome, insulin-dependent diabetes mellitus, and the post-polio syndrome. Enteroviral persistence has been demonstrated *in vitro*. However, as in the case of enteroviral persistence in neonates and patients with agammaglobulinaemia, these are chronic productive infections. This is in contrast to enteroviral persistence as supposed to occur in the diseases as mentioned above, in which no virus can be isolated, and in which only the viral RNA has been found. The following paragraphs will review the literature associating enterovirus infections with several chronic medical conditions, to investigate whether there is indeed evidence for enteroviral persistence in these diseases.

Idiopathic dilated cardiomyopathy

In idiopathic dilated cardiomyopathy (IDC), the heart chambers are dilated. The aetiology of this disease is unknown. Incidences of 5 to 10 cases per 100 000 people per year have been reported from the USA and Sweden.^{6,7} The disease has a variable clinical course without useful prognostic markers. As a result, it has been difficult to evaluate the effect of medication and the only effective therapy for end-stage disease is heart transplantation. In fact, it is the second most common reason for cardiac transplantation in the UK.⁸

Epidemiological studies revealed that enterovirus (mainly Coxsackie B) is one of the most important causative agents of acute myocarditis.⁹ It is thought that

Table 1. Molecular detection of enteroviral RNA in chronic diseases

Reference	Country	Method	Disease	Number of patients	Enteroviral positive
Bowles <i>et al.</i> ^[13]	UK	QSB	IDC	21	6
			Control	19	1
Bowles <i>et al.</i> ^[14]	UK	QSB	IDC	8	5
			Myoca	9	4
			Control	5	0
Archard <i>et al.</i> ^[15]	UK	QSB	IDC	29	16
			Myoca	21	12
			Control	22	0
Wiegand <i>et al.</i> ^[16]	G/USA	DS	IDC	6	1
			Control	8	0
Cochrane <i>et al.</i> ^[17]	UK	NB	IDC	19	0
			Control	21	0
Kandolf <i>et al.</i> ^[18]	G	ISH	IDC		
			recent	33	10
			chronic	48	8
			Myoca	62	13
			Control	53	0
Weiss <i>et al.</i> ^[19]	USA	PCR	IDC	11	0
			Myoca	5	1
			Control	21	0
Weiss <i>et al.</i> ^[20]	USA	PCR	IDC	11	5
			Control	24	9
Zoll <i>et al.</i> ^[21]	NL	PCR	IDC	5	1
			Control	8	0
Petitjean <i>et al.</i> ^[22]	F	PCR	IDC	45	30
			Myoca	10	3
			Control	23	9
Grasso <i>et al.</i> ^[23]	I	PCR	IDC	21	0
			Control	20	0
Jin <i>et al.</i> ^[24]	USA	PCR	CMP	23	4
			Myoca	25	1
Keeling <i>et al.</i> ^[25]	UK	PCR	IDC	50	6
			Control	75	13
Liljeqvist <i>et al.</i> ^[26]	S	PCR	IDC	35	0
			Control	23	0

UK, United Kingdom; G, Germany; USA, United States of America; NL, Netherlands; F, France; I, Italy; QSB, Quantitative slot-blot; DS, Dot-spot; NB, Northern-blot; ISH, *In situ* hybridisation; PCR, Polymerase chain reaction; IDC, Idiopathic dilated cardiomyopathy; Myoca, Myocarditis; CMP, Cardiomyopathy.

IDC may be a late outcome of viral myocarditis.¹⁰ It is also assumed that IDC develops after the virus has been eliminated, and therefore it is suggested that the virus triggers an autoimmune response.^{11,12} Over the past few years it has been suggested that enteroviruses are able to persist and that persistence could play a role in the aetiology of myocarditis and IDC. The data are summarised in Table 1. Detection of viral RNA in specimens from patients, while the virus itself can no longer be isolated, led investigators to suggest that the viral RNA persists in some patients and predisposes to the maintenance of disease.

Although enteroviral RNA could be detected in cardiac biopsies from patients with myocarditis (20%–50%) or IDC (0%–75%), the incidence of positive patients

varies significantly between the different studies. *In situ* hybridisation (ISH) studies revealed that the enteroviral infection has a highly focal character.²⁷ Since, in practice, only few (or even one single) biopsies are tested per patient for enteroviral RNA detection, sampling error can explain part of the variation. However, this is not the most plausible explanation since most studies have used explanted hearts, which contain enough material for reliable determination.

The molecular method which was used for the detection of enteroviral RNA varies from study to study (Table 1). Kandolf *et al.*¹⁸ used ISH with enteroviral probes and detected enteroviral RNA in 22% of the patients with IDC. Cronin *et al.*²⁸ reported that slot-blot hybridisation sometimes revealed unexplained strong

hybridisation signals and that slot-blot hybridisation is less reliable than ISH. It can therefore not be excluded that the results, obtained by slot-blot hybridisation, are at least in part caused by cross-hybridisation with unrelated sequences. PCR is considered to be the most sensitive technique for the detection of enteroviruses today.²⁹ Furthermore, the selection of highly specific primers and oligonucleotide probes will result in a specific amplification of enteroviral target sequences. It has indeed been shown that PCR is more specific than slot-blot hybridisation.²⁴ Using PCR, several groups^{19,23,26} failed to detect enteroviral RNA in biopsy samples from patients with IDC, while Petitjean *et al.*²² found 75% of the patients with IDC positive for enteroviral RNA. The results of Weiss *et al.*¹⁹ may be explained by the fact that they only used a coxsackievirus B3 specific PCR assay, which implies that they may have missed other enterovirus serotypes possibly involved in the disease. In fact, very recently, the same authors described a 45% positivity rate in patients with IDC using highly conserved enterovirus specific primers, although they also detected enteroviral RNA in 38% of the control specimens.²⁰ Petitjean *et al.*²² also found 39% of the control group (i.e. patients with cardiomyopathy without viral aetiology) positive for enteroviral RNA, possibly indicating that the presence of enteroviral RNA in heart muscle tissue is more common than has been appreciated. Similar findings are reported by Keeling *et al.*,²⁵ who also described a similar prevalence of enteroviral RNA in control patients (17%) and patients with IDC (12%), and suggested that enteroviruses are common infectious agents rather than the causative agents of IDC. The fact that enterovirus seems to be common in heart muscle is also described by Easton and Eglin,³⁰ who detected enteroviral RNA in 46% of the myocardial biopsies from a selected group of patients with proven coxsackievirus B infection at the time of death.

Various animal models have been described of picornavirus induced myocarditis,³¹ of which the murine model of coxsackievirus B3 induced myocarditis is the most extensively studied. This model resembles the human disease in that chronic inflammation of heart tissue can often be observed for months after infection. Klingel *et al.*³² described an ongoing myocarditis in this murine model related to the persistence of coxsackievirus B3. Although the virus could not be isolated after 15 days p.i., the viral RNA could still be detected up to 30 days p.i. by ISH. It was suggested that the enteroviral RNA can persist and contribute to the maintenance of disease.³²⁻³⁴ Immune-mediated processes may be triggered and maintained by enteroviral RNA persistence and the viral RNA might not be eliminated after initiation of the disease. However, in the acute phase of disease 13% of the myocardial cells were found to be infected, while at day 30 p.i., only 0.01% of the myocardial cells were found positive for enteroviral RNA. Koidi *et al.*³⁵ detected enteroviral RNA up to day 28 after inoculation. They describe a decreasing number of positive animals with time so that by day 28, the virus could no longer be detected in myocardial cells. Similar results have been

described by Cronin *et al.*²⁸ Using an encephalomyocarditis virus-induced myocarditis (and myositis) murine model, infectious virus could be isolated up to 2 weeks p.i. The viral RNA could, however, be demonstrated by ISH for up to 3 weeks but not thereafter. These data together support the idea that the infection is dying out, rather than persisting. Jin *et al.*²⁴ described a case of a patient with myocarditis, positive for enteroviral RNA by PCR, who was found again positive 3 months later. A third biopsy, 4 months thereafter was, however, negative. This could be explained either by sampling error or by the fact that the viral RNA was gradually being eliminated.

Idiopathic inflammatory myopathies

Polymyositis (PM) is an inflammatory disease of the skeletal muscle.³⁶ It is the most frequently acquired muscular disease in adults and has a chronic progressive character. Only half of the patients have long lasting responses after treatment with antiinflammatory and immunosuppressive drugs. Despite treatment, many patients become progressively handicapped. Dermatomyositis (DM) affects both adults and children and can be identified by a characteristic rash which often precedes muscle weakness.³⁷ Muscle weakness, electromyographic findings and elevated muscle enzymes are equally present in both inflammatory myopathies.³⁶ Inclusion body myositis (IBM) is considered to be a distinct type of myositis. IBM presents frequently as polymyositis-like disease with onset above 50 years and resistance to therapy.³⁸

Several findings suggest a relation between enteroviruses and myopathy. Enterovirus has sporadically been isolated from muscle biopsies of patients with PM.^{39,40} Using electron microscopy, virus-like crystals have been detected in muscle tissue,⁴¹ but these crystals could have been β -glycogen particles.⁴² It has also been described that chronic enterovirus infections in patients with agammaglobulinaemia are often associated with DM-like symptoms.³ However, in contrast to PM and DM, enterovirus can still be isolated in these chronic infections.

To investigate the hypothesis that persistence of enterovirus is involved in the maintenance of PM, DM and IBM, several research groups have used molecular hybridisation to detect the enteroviral genome in skeletal muscle (Table 2). In none of a total of 53 patients with IBM, could viral RNA be detected (Table 2). In the case of PM and DM the results are less consistent. Using quantitative slot-blot analysis Bowles *et al.*⁴³ and Cunningham *et al.*⁴⁴ demonstrated enteroviral RNA in a significant number of patients. Yousef *et al.*⁴⁵ confirmed these results for PM patients with ISH. These results were not confirmed by Rosenberg *et al.*,⁴⁶ using ISH, but this group detected a positive signal in 3 of the 9 cases with DM when a probe for Theiler's encephalomyelitis virus (a cardiovirus) was used. A strong sequence homology has been described between cardioviruses and eukaryotic enzymes such as tRNA synthetases.⁵⁰ However, this was based on amino acid sequences and it does not

Table 2. Molecular diagnosis of enteroviral RNA in chronic diseases

Reference	Country	Method	Disease	Number of patients	Enteroviral positive
Bowles <i>et al.</i> ^[43]	UK	QSB	PM	2	1
			DM	7	4
			Control	10	0
Cunningham <i>et al.</i> ^[44]	UK	QSB	PM	46	9
			DM	36	14
			IBM	24	0
			Control	128	0
Yousef <i>et al.</i> ^[45]	UK	ISH	PM	11	6
			DM	2	0
			Control	13	0
Rosenberg <i>et al.</i> ^[40]	USA	ISH	PM	5	0
			DM	9	3*
			IBM	5	0
			Control	14	0
Leff <i>et al.</i> ^[47]	USA	PCR	PM	18	0
			DM	12	0
			IBM	14	0
			Control	13	0
Leon-Monzon and Dalakas ^[48]	USA	PCR	PM	16	0
			DM	12	0
			IBM	11	0
			Control	16	0
Jongen <i>et al.</i> ^[49]	NL	PCR	PM	10	0
			DM	5	0
			Control	15	0
Gow <i>et al.</i> ^[67]	UK	PCR	PVFS	60	32
			Control	41	6
Archard <i>et al.</i> ^[66]	UK	QSB	PVFS	96	20
			Control	4	0
Cunningham <i>et al.</i> ^[44]	UK	QSB	PVFS	140	34
			Control	152	0
Melchers <i>et al.</i> ^[70]	NL	PCR	PPS	16	0
			Control	7	0

See Table 1; PM, Polymyositis; DM, Dermatomyositis; IBM, Inclusion body myositis; PVFS, Post viral fatigue syndrome; PPS, Post-polio syndrome. *Only positive for TMEV.

necessarily reflect the RNA sequences. On the other hand, however, the positive signal was not detected in the muscle fibres itself.⁴⁶ Although PCR is more sensitive than slot-blot hybridisation, Leff *et al.*,⁴⁷ Leon-Mozon and Dalakas,⁴⁸ and Jongen *et al.*⁴⁹ were not able to demonstrate the presence of enteroviral RNA in these patient groups. Again, it cannot be excluded that the results obtained by slot-blot hybridisation are at least in part due to cross-hybridisation with unrelated sequences.²⁸

The most widely used animal model for polymyositis is the coxsackievirus B1 induced murine myositis.⁵¹ As with myocarditis, the model resembles the human disease in that affected animals develop a chronic myositis in the hamstrings. The virus can be isolated from the muscles until 14 days p.i.^{52,53} Beyond this time the virus can no

longer be isolated, although RNA can still be detected by molecular hybridisation. Tam *et al.*⁵² were only able to detect the viral RNA up to about 28 days p.i. These results were confirmed by Zoll *et al.*⁵³ using PCR. No viral RNA could be detected thereafter. Both a myocarditis and a myositis could be induced in mice by a myotropic variant of encephalomyocarditis virus.²⁸ Virus could be isolated in high titres at 7 days p.i., but only a very low titre was found at 2 weeks p.i., and no virus could be isolated thereafter. Using ISH, positive hybridisation was detected in skeletal muscle at 3 weeks but was only seldomly found as late as 4 weeks p.i. Similar to the coxsackievirus B induced murine cardiomyopathy model as described above, this suggests that the infection is dying out after 4–6 weeks rather than persisting.

Insulin-dependent diabetes mellitus

Insulin-dependent diabetes mellitus (IDDM) is the result of a progressive destruction of pancreatic β -cells.⁵⁴ It is considered to be an autoimmune disease in which, apart from genetic factors, environmental factors such as virus infections might play a role.⁵⁴

The association with enterovirus (Coxsackie B) is suspected on the basis of several findings. Case reports have described the development of diabetes mellitus, shortly after an infection with Coxsackie B virus,⁵⁵ and epidemiological studies showed that IgM antibodies were found more frequently in patients with diabetes mellitus than in control groups.^{56,57} A possible role of the virus in the destruction of the pancreatic β -cells remains unclear. The viral infection may trigger a β -cell specific autoimmune response. Another possibility is that the viruses infect the insulin-producing β -cells and persist in these cells. However, a viral infection is only found rarely at the moment IDDM is diagnosed. In none of the 88 autopsy pancreases from patients who died of diabetes mellitus, could VP1 antigens be detected.⁵⁸ However, this cannot be regarded as evidence against the hypothesis of enteroviral persistence, since VP1 might not be expressed during the persistence of viral RNA. In fact, Kuge *et al.*⁵⁹ reported that deletions in the genomes of DI particles derived from the Sabin strain of poliovirus type I, were limited to the internal genome region encoding the viral capsid proteins. These facts do not exclude an aetiological role for viruses, but a possible relationship must be complex.

Recently, a model has been described in which infection of mice with coxsackievirus B4 led to a chronic infection of the pancreas.⁶⁰ The virus titres in the pancreases were low, and the animals had a continuing humoral immune response, but infectious virus could still be isolated for up to 10 months. However, these experiments were performed with a pancreas adapted variant of coxsackievirus B4 which suppresses MHC class I expression. On the other hand, this again reflects a chronic productive infection in which the virus could be isolated at any time of the infection and is therefore different from latency as supposed by the single finding of viral RNA.

On the basis of the data described so far, possible enteroviral RNA persistence can not be excluded, but it has certainly not been proven.

Post-viral fatigue syndrome

The post-viral fatigue syndrome (PVFS) is considered to be a distinct form of the chronic fatigue syndrome, with onset after a flu-like episode with fever which is believed to be of viral origin.^{61,62} PVFS is characterised by an excessive disabling fatigue which persists for more than 6 months.⁴⁴ The fatigue is accompanied by diverse physical complaints.⁴⁴ A relationship with enteroviruses is suspected mainly on (sero)epidemiological findings. High levels of neutralising antibody against Coxsackie B viruses were found in 50% of the PVFS patients as compared with 17% of healthy controls.⁶³ McCartney *et al.*⁶⁴ detected Coxsackie B virus specific IgM in 31% of the patients. Because virus specific IgM responses were

detected in consecutive sera from several PVFS patients for a year or even longer, it was suggested that this is indicative for viral persistence rather than a recent infection.⁶⁴ Further indication for viral persistence was reported by Yousef *et al.*,⁶⁵ who found infectious enterovirus in faecal specimens of about 20% of the patients with PVFS after dissociation of immune complexes, whereas direct virus isolation failed. A few groups have used molecular detection to investigate whether enteroviral RNA is present in skeletal muscle of PVFS patients (Table 2). Using quantitative slot-blot hybridisation, Archard *et al.*⁶⁶ and Cunningham *et al.*⁴⁴ found approximately 25% of the muscle biopsy samples positive for enteroviral RNA. None of the controls was positive. Gow *et al.*,⁶⁷ using PCR, confirmed these data and found 53% of the patients positive for enteroviral RNA. However, they also found 15% of the controls positive for enteroviral RNA, which is highly exceptional. Based on these results the authors suggested that enteroviral persistence may play a role in the pathogenesis of the PVFS.^{44,66,67}

However, before it can be stated that enterovirus indeed can persist in muscle from patients with the PVFS, these results should be confirmed by other groups. All studies on molecular detection of enteroviral RNA in PVFS were performed in patients from the UK, and may reflect a particular approach of selecting patients, a geographical difference, or technical aspects of enterovirus detection. Therefore it would be interesting to compare data from the UK systematically with those of other countries, as can be done for idiopathic inflammatory myopathies, in which, from the limited data available, enteroviral RNA was only detected in muscle biopsies of patients from the UK.

Post-polio syndrome

As is the case with the diseases discussed before, the pathogenetic mechanism underlying the post-polio syndrome (PPS) is not understood. The syndrome manifests itself about 30 years after an attack of paralytic poliomyelitis and a relationship with the previous poliovirus infection is suspected.⁶⁸ An association with poliovirus persistence was suspected because intrathecal synthesis of IgM antibodies to poliovirus was found in patients with PPS.⁶⁹ However, these findings could not be confirmed. Using PCR, we and others failed to detect poliovirus RNA in muscle samples and cerebrospinal fluid from patients with the PPS.^{48,70} Neither could we detect poliovirus specific IgM antibody in the cerebrospinal fluid of these patients. The basis for possible enteroviral persistence is very small but as long as no brains and peripheral nervous tissues have been investigated, persistence can not absolutely be excluded.

CONCLUDING REMARKS

Viral persistence can be divided into chronic productive infections, in which infectious virus is present and can be

recovered by conventional methods, and into latent infections. In the latter, the viral genome is present but infectious virus is generally not produced except during intermittent episodes of reactivation.⁷¹ Persistent enterovirus infections in neonates or patients with agammaglobulinaemia are of the chronic productive type. Enteroviral persistence, as is supposed to occur in the chronic diseases IDC, PM/DM, IDDM, PVFS and PPS, is more difficult to classify. These infections might be considered to be latent since the viral RNA is detected while the virus cannot be isolated.

In the mouse models of IDC and PM/DM the viral RNA can be detected for about 4 weeks p.i. Since virus can be isolated for 2 weeks p.i., this means that the virus 'persists' only for 2 additional weeks. It is furthermore difficult to envisage how the viral RNA could be maintained in a cell without expression of viral products, which so far has not been experimentally described in the 'persistent' state of infection. Hence, the hypothesis of enteroviral persistence is not well founded.

As mentioned before, enteroviruses cause a lytic infection. The infection is controlled by the immune system which will become active within 5–7 days. For reasons which are not fully understood the lytic infection can slow down to a chronic productive one with little or no cell lysis, as has been demonstrated *in vitro*.^{72,73} So it may be that the virus can be trapped in a few cells that, probably because of defective virus replication, do not immediately lyse. Several reports have described an abnormal production of equal amounts of positive and negative strands of enteroviral RNA in patients with the PVFS as well as in coxsackievirus B3 induced myocarditis in the mouse.^{32,74,75} This double-stranded RNA intermediate is very stable and not as sensitive to RNase degradation as the single-stranded viral genome. The presence of a 1:1 molar ratio of the (+) and (–) strand RNA implies that all (+) strand viral genomes are captured in a highly stable complex, so that further production of infectious virus will be prevented. The RNA will eventually be eliminated. In the murine models this occurs within 4 weeks, in the human situation probably after a long period of time. This means that the infection is dying out.

The literature concerning the presence of enteroviral RNA in humans is conflicting (see Tables 1 and 2). Part of the controversy may be explained by sampling errors or the unreliability of conventional hybridisation assays as slot-blot hybridisation.^{24,28,48} It would be convincing if enteroviral antigens were also found, and if repeated biopsies were tested to follow persistence in time.

Does this all mean there is no place for enteroviruses in the pathogenesis of the human chronic diseases? There is still evidence for a relationship, which is also supported by the murine models. It only implies that a sustained presence of the viral RNA in the affected tissues may not be essential. Post infectious auto-immunity has been postulated by many investigators and remains an important issue to be studied.^{11,76,77} From such studies in the murine cardiomyositis model it became clear that anti-

heart antibody is generated in the myocarditic mouse,¹¹ that autoreactive cytotoxic T lymphocytes that can cause cardiac damage after adoptive transfer to uninfected mice are generated as well.⁷⁷ In addition, several epitopes have been described that show cross reactivity between Coxsackie B viruses and cardiac myocytes (molecular mimicry).^{11,12} The strain of Coxsackie B virus used, as well as genetic factors of the host, are found to be of crucial importance. What makes a strain myocarditic or not is still poorly understood. The difference might reside in the type of immune response mounted by the various strains.⁷⁸ In this paper, the present literature concerning persistence of enteroviruses as a cause for the development of human disease was reviewed, and we conclude that there is no clear evidence for enteroviral RNA persistence and the subsequent development and maintenance of chronic medical conditions in humans.

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