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FORUM

There is Evidence for Persistent Enterovirus Infections in Chronic Medical Conditions in Humans

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Enteroviruses are members of the *Picorna*virus (*smallRNA* virus) family. These viruses have been studied extensively over many years; in cultured cells, in animal models and in human disease. Early experiments on the growth of poliovirus in cultured primate epithelial cells revealed that cellular DNA and RNA synthesis are inhibited profoundly while enteroviral RNA and processed viral proteins accumulate rapidly. Large amounts of infectious and defective progeny are assembled exponentially over a few hours, resulting in extensive cytolysis and the animal virus analogy closest to a bacteriophage 'burst'.[1] A similar picture was seen in human disease[2] where the most easily recognisable scenario was enteric infection by the faecal–oral route, sometimes with spread via the lymphatic system leading to viraemia and transient, febrile illness but almost always self-resolving without sequelae and resulting in good humoral immunity.

This early concept of exclusively acute, cytolytic infection by enteroviruses has remained in the minds of many. Unlike infection with some other clinically important viruses such as the human herpesviruses or retroviruses, there seemed to be no fundamental mechanism of latency or persistence inherent in enterovirus replication.

Happily, the structure, organisation, replication and expression of the virus genome are now very well understood in enteroviruses, largely as a result of the application of various molecular biological techniques.[3*passim*] This is because these small simple viruses lend themselves to manipulation and analysis in a way which is hardly possible with larger, more complex (DNA) viruses. Picornaviruses short-circuit the usual role of DNA in the hierarchy of genetic information transfer and replicate solely from an RNA genome: they can be propagated in the presence of inhibitors of DNA-dependent RNA synthesis and even in anucleated cells. The genome is a single molecule of about 7400 ribonucleotides and the complete genomic sequence of various enteroviruses has been determined by a range of strategies.[4–7] The genome is structurally and functionally analogous to a eukaryotic mRNA and is translated monocistronically to a precursor polyprotein which is then cleaved site-specifically, yielding the virus gene products in equimolar amounts.[8] There is a large (>700 nucleotides) 5′ non-translated region which contains both conserved and variable sequence motifs[9] by which a particular enterovirus can be identified. The 3′ terminus is polyadenylated, a feature which, together with random oligonucleotide priming, has allowed the synthesis, molecular cloning and nucleotide sequencing of full-length cDNA.[7] These clones can be manipulated *in vitro* and modified infectious virus resurrected from them by transfection of cultured cells with viral cRNA, transcribed *in vitro* from recombinant vectors containing appropriate promoters: such modified virus can then be tested in animal models to analyse the molecular basis of changes in phenotype, for example, tissue tropism or virulence.[10]

Molecular hybridisation techniques using cloned enterovirus-specific sequences as probes[11*passim*] and reverse transcription PCR[12,13] have greatly enhanced the ability to detect viral RNA sequences in tissue samples from experimentally infected animals or patients. More recently, we have employed two nested pairs of enterovirus primers to increase sensitivity and specificity, combined with subsequent nucleotide sequencing to confirm the identity of the PCR product.[14] There is a comprehensive literature demonstrating that it is frequently possible to detect persisting enteroviral nucleic acid sequences in clinical samples under circumstances in which infectious virus cannot be isolated nor virus-specific antigens revealed by immunofluorescence.[15,11]

PERSISTENT INFECTION IN CELL LINES AND EXPERIMENTAL ANIMALS

As in all basic medical research, there are lessons to be learnt from *in vitro* and animal model experiments and

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Abbreviations used: DCM, dilated cardiomyopathy; PVFS, post-viral fatigue syndrome.

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these will be considered before discussing chronic human enteroviral disease. Far from an exclusively cytolytic replication cycle resulting in acute infection, there are many examples of persistent enterovirus infection in cultured cells and animal models which are relevant to human disease. Coxsackie group B viruses readily establish persistent infections in human lymphoid cell lines.[16] Coxsackie virus B3 replicates in human myocardial fibroblasts over prolonged periods at a rate which can be modified by interferon[17] and has been employed in various mouse models of enteroviral heart muscle disease. This virus produces multi-system disease in many strains of mouse including the ubiquitous [18,19] Balb/c but exclusively a dose-dependent myocarditis in SWR/J mice.[10] Coxsackie B3 has been shown by molecular techniques to persist in mouse myocardium in various models for up to 56 days[19–23] and in hamster for up to 180 days.[23] The same serotype establishes persistent infection of human fetal pancreatic islet-like cells in culture[24] and experimental pancreatitis in mouse.[25,26] Variants of the conceptually similar encephalomyocarditis virus produce insulin-dependent diabetes in the mouse[27] and the sequence changes conferring this phenotype have been mapped.[28] Persistent infection of a human rhabdomyosarcoma cell line (i.e. originating from skeletal muscle) with Coxsackie virus B5 is well established[29] and the virus infection modifies expression of some cellular genes. There is a mouse model of Coxsackie virus B1-induced polymyositis[30] in which virus RNA has been detected by PCR at 12 months post infection.[31] Persistence of poliovirus has been shown in Hep-2 and Human erythroblastoid cells[32,33] but not yet in human cells originating from the CNS. Asymptomatic infection of mouse brain with poliovirus type II was demonstrable by conventional virological techniques for 77 days.[34] Although a mouse rather than a human pathogen, persistent infection with Theiler's encephalomyelitis virus has been demonstrated in cultured glioma cells[35] and brain macrophages[36] and experimental animal infection is associated with chronic demyelination.[37] These are powerful indicators of a role of enteroviruses in inflammatory and post-inflammatory disease of heart and skeletal muscle and of the CNS and are difficult to discount although, in some chronic inflammatory diseases, virus infection may be only the initiator of a subsequently autoimmune disorder.

PERSISTENT INFECTION IN HUMANS

The best evidence of persistent enteroviral infection pursuant with chronic human illness comes from heart muscle disease (myocarditis and dilated cardiomyopathy; DCM). It is widely accepted that Coxsackie B viruses are aetiological agents of myocarditis.[38] This association was established originally by retrospective serology[39] and confirmed subsequently by nucleic acid hybridisation[40–43] and by PCR[12–14] using enterovirus group-specific probes and primers to detect virus RNA in myocardium. This is despite the fact that infectious virus could be isolated from endomyocardial biopsy samples only rarely and at an early stage of disease.[15 *passim*] It was suggested that DCM, which leads to progressive congestive heart failure and is one of the major indications for cardiac transplantation, is a sequela of a previous (viral) myocarditis.[44,45] This is supported by continuing enterovirus monotypic IgM responses for up to 10 years in patients with chronic relapsing pericarditis or dilated cardiomyopathy[46,47] and confirmed by hybridisation studies on tissue from explanted hearts which show that enterovirus can persist in the myocardium of patients with DCM until end-stage disease requiring cardiac transplantation.[48] Indeed, persistence of enterovirus in the myocardium of patients with DCM, some with many years history of heart muscle disease, is a powerful independent predictor of poor prognosis, indicating causality.[49] The identity of PCR products, amplified from myocardium of patients with DCM by using enterovirus group-specific primers, has been established by direct nucleotide sequencing, and shown to be most closely related to Coxsackie B3 in all cases tested.[14] These studies indicate that Coxsackie viruses can persist in myocardium through all stages of the progression from acute myocarditis to end-stage congestive heart failure and have a causal role in disease.

There is good evidence that a similar pathogenetic process occurs in skeletal muscle with progression from acute inflammatory disease (myositis), which may become chronic with elements of autoimmunity, to a post-inflammatory situation with little or no muscle histopathology but chronic excessive muscle fatiguability on moderate exercise (post-viral fatigue syndrome; PVFS). Enterovirus sequences were detected by slot blot[50] or *in situ* hybridisation[51] in muscle biopsies from patients with polymyositis or juvenile dermatomyositis. One PCR study failed to confirm this[52] although there are positive PCR results, confirmed by nucleotide sequence of the amplified product (see below).

While the proposed aetiologies of chronic fatigue syndromes are legion, highly selected patients presenting with *excessive muscle fatiguability* and a good premorbid personality often date their illness to a 'flu-like, presumed viral, episode from which they failed to recover completely. Enterovirus was isolated, after acid dissociation of neutralising antibody, from the stools of some such patients and the same serotype shown to be present in a proportion on retesting one year later.[53] Skeletal muscle biopsies from 20 of 96 (21%) PVFS patients, with up to 20 years history of fatiguability were positive for enterovirus RNA in quantitative slot blot hybridisations using a group-specific probe.[54] Hybridisation data from a further series of patients show enterovirus sequences in muscle biopsies from 41 of 158 (26%) PVFS patients and 25 of 96 (26%) histologically proven cases of inflammatory muscle disease, compared with only 2 of 152 (1·3%) normal or pathologically irrelevant controls.[55]

A PCR study found 53% of PVFS muscle biopsies positive compared with 15% of controls although nucleotide sequence data were not presented.[56] However, the same group found no significant difference between a further PVFS group and a comparison group of patients

with other neuromuscular disorders.[57] Nucleotide sequence data from the PCR products of a nested two-stage reaction, sufficient to identify the enterovirus serotype involved, are available from a small number of biopsies from PVFS patients (LCA; unpublished data) and establish the persistence of enterovirus in skeletal muscle.

The evidence for persisting enterovirus involvement in chronic diseases of the human CNS is less well established but intriguing and these deserve further investigation. For example, chronic meningoencephalitis is recognised in hypogammaglobulinaemic patients[58,59] and enterovirus RNA was detectable in CSF by PCR over a 2 year period, even when culture negative.[60,61] Similarly, although one report describes failure to detect enterovirus RNA by PCR in muscle from patients with post-polio syndrome,[62] more recent investigations have shown enterovirus RNA sequences in CSF and in tissue from CNS.[63–65] Some such patients have poliovirus-specific IgM CSF and evidence of an intrathecal T cell response.[66]

MECHANISMS OF ENTEROVIRUS PERSISTENCE

Having considered the experimental and clinical evidence for persisting enterovirus infection, we wish now to consider possible mechanisms. It seems that enterovirus persistence in most cell culture systems involves steady state, low level productive infection which may involve only a minority of the cell population which may have an altered phenotype.[16] It is difficult to extrapolate from cell culture systems to animal models or clinical situations but this seems to be the case in Theiler's virus infection of mouse CNS.[67] There is evidence that the enterovirus itself evolves in persistently infected cell cultures, perhaps becoming less replication competent.[68] Enterovirus persistence in clinical situations has been shown to result from a different mechanism in which replication defective virus mutants emerge, presumably during the initial phase of disease, and no longer produce infectious progeny or the normal range of processed viral antigens. Thus, acute cytolytic inflammatory disease progresses to persistent infection with defective virus, no longer cytolytic and no longer attracting the attention of the host cellular immune response. Single-stranded RNA viruses accumulate sequence mutations rapidly, partly due to the high error rate of the RNA-dependent RNA polymerase ($1–5 \times 10^{-3}$) and partly because there is no template against which to correct a mismatched nucleotide. Loss of specificity of the viral RNA polymerase is an altered function seen in persistent enterovirus infection in post-inflammatory heart muscle disease[11,69,19] and skeletal muscle disease.[70] Instead of the (−) template strand being copied selectively to generate and excess of (+) genomic strand RNA, the polymerase replicates both strands equally, presumably as a result of mutation in the polymerase gene or in its recognition sequence. This might be expected to lead to annealing of complementary viral RNAs and inhibition of translation of viral proteins by cellular ribosomes. Double-stranded viral RNA is an efficient inducer of interferon which itself may affect productive virus replication.[17,71] Of course, accumulating sequence mutations in enterovirus RNA may affect other regions of the genome such as the regulatory functions of the 5′ non-translated region, the amino acid sequence of structural proteins[68] or even the recognition sequences for proteolytic cleavage of the precursor polyprotein[72] leading to failure to process and display the normal range of viral antigens. The last is difficult to address experimentally as diagnostic or convalescent sera contain antibodies directed against only a limited range of epitopes and so western blotting is not informative. Whatever the mechanism of enterovirus persistence, it is worth asking how this might cause disease. While persistent virus infections in general are known to modify the expression of some host cell genes,[73] little is known about the pathogenesis of chronic enterovirus infections and this would not necessarily be the same in the various disease processes discussed above. However, a recent study showed a positive correlation of premature, profound lactic acidosis on subanaerobic threshold exercise testing of some chronic fatigue syndrome patients with the presence of enterovirus RNA sequences in their non-inflammatory muscle biopsies.[74] It seems that these patients had, in effect, an acquired mitochondrial myopathy as a result of persistent enterovirus infection.

In conclusion, there are many indications from direct investigations and by analogy that persistent enterovirus infections are, in fact, causally associated with chronic medical conditions in humans. The requirement now is not for debate but for more directed research into mechanisms of virus persistence and pathogenesis, hopefully opening the door to rational attempts at prevention and therapy.

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