Acute enterovirus infection followed by myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) and viral persistence

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ABSTRACT

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Accepted 1 September 2009 Published Online First 14 October 2009 **Aims** Enteroviruses are well-known causes of acute respiratory and/or gastrointestinal infections and nonspecific flu-like illness. Although enterovirus protein, RNA and non-cytopathic viruses have been demonstrated in the stomach biopsies of patients with myalgia encephalomyelitis/chronic fatigue syndrome (ME/CFS), causality for chronic diseases is difficult to establish without having well-documented cases of acute enterovirus infections. The aim of this study was to link acute enteroviral infection to viral persistence in patients with ME/CFS.

Method Patients admitted to the hospital with acute febrile illnesses were screened for enteroviral infections. Acutely infected patients were followed longitudinally, and those who developed symptoms of ME/CFS underwent oesophagogastroduodenoscopy and biopsies of the antrum to document viral persistence by immunoperoxidase staining for viral protein and viral RNA assay. **Results** Three representative patients with different manifestations of acute enterovirus infections progressed to have chronic symptoms of ME/CFS. Persistent viral infection was demonstrated in the antrum years later. Conclusion After acute infections, enteroviruses can persist in patients resulting in manifestation of ME/CFS. Chronic enterovirus infection in an immunocompetent host may be an example of a stalemate between attenuated, intracellular viruses and an ineffective immune response.

Acute enterovirus infections affect more than 30–50 million people in the USA yearly.¹ Although many cases are asymptomatic, significant numbers of patients develop upper and/or lower respiratory or gastrointestinal infections or non-specific flu-like illness, with viral dissemination to brain, heart, muscles and many other organs. Most experts believe that enterovirus infection would resolve without developing chronic persistence except in patients.² severely immunocompromised Mounting evidence from animal models suggests, however, that chronic persistent infection can occur in immunocompetent mice. Recent experimental data from murine models of coxsackievirus B3 myocarditis demonstrated that a naturally occurring alteration to an enteroviral genome, in the form of deletion in the 5' untranslated region, is associated with long-term persistence.⁴ A similar finding has been demonstrated in one patient who died of coxsackievirus B2 myocarditis.⁵

Persistent enterovirus infection has been implicated in a number of chronic human diseases including, but not limited to, dilated cardio-

myopathy, chronic muscle disorders, type 1 diabetes mellitus and myalgia encephalomyelitis/chronic fatigue syndrome (ME/CFS).^{1 4 6} A severe flu-like illness that occurred in the majority of cases of ME/ CFS, followed by persistent illness and fatigue, suggests infectious aetiologies triggering and possibly perpetuating this syndrome.⁷ A number of welldocumented acute viral or bacterial infections can be followed by the manifestations of ME/CFS. $^{\rm 8-11}\,\rm Our$ recent study demonstrated the presence of enterovirus protein, viral RNA and growth of non-cytopathic viruses from stomach biopsies taken from ME/CFS patients, years after acute flu-like illness.¹² However, it has been difficult to establish causality for chronic diseases without having well-documented cases of acute enterovirus infections. We diagnosed several cases of acute enterovirus infection and followed the patients longitudinally over a few years. Three patients fulfilled the criteria for ME/ CFS, and the presence of viral persistence was demonstrated in the stomach biopsies years later.

MATERIALS AND METHODS

Patients admitted to the local community hospitals with severe respiratory and/or gastrointestinal (GI) infections or non-specific febrile illnesses were screened for the presence of enterovirus infections. Assay for enterovirus RNA was performed on the infected secretions (ie, tracheal secretions, perirectal swab, peripheral blood leucocytes (PBLs) and/or the biopsied tissue specimens, within first month of the illness or at a specified date). Of the patients who developed prolonged symptoms fulfilling the criteria for ME/CFS and had persistent GI symptoms, oesophagogastroduodenoscopy was performed years later with three biopsies taken from the antrum. The biopsies were assayed for enterovirus RNA and stainable protein.

Enteroviral RNA testing

PBLs were harvested from 10 ml of whole blood within 2 h of sampling, and the RNA was extracted using Qiagen Blood RNA Mini Kit (Qiagen, Valencia, California, USA). A 250 μ l volume of supernatant of the centrifuged tracheal secretion was placed in 750 μ l TriZol LS (Invitrogen, Carlsbad, California, USA), and the RNA was extracted according to the manufacturer's instructions. Four sections (10 μ m) of the decalcified, paraffinised bone marrow biopsy specimen were processed with PureLink FFPE RNA isolation kit (Invitrogen). Antrum biopsies preserved in RNA-later were homogenised in 1.5 ml TriZol LS. RNA was extracted from 250 μ l of the suspension according to manufacturer's instruction.

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Extracted RNA was amplified with biotinylated primers provided by pan-enterovirus oligo-detect kit and sequenced using non-biotinylated primers as described.¹²

Real-time qRT-PCR was carried out using Master cycler and enterovirus RNA primers (Cepheid, Sunnyvale, California, USA) and a quantitative enterovirus RNA control (Ambion Diagnostics, Austin, Texas, USA) according to the manufacturers' instructions. The number of enterovirus RNA copy was determined by estimating from the enterovirus RNA standards.

Immunoperoxidase staining of the paraffin-embedded antrum or other tissues was performed with an enterovirus-specific murine monoclonal antibody (5D8/1; Dako, Carpenteria, California, USA) or a cytomegalovirus mAb with the same isotype (Chemicon, Temecula, California, USA) as a negative control. Standard staining protocol was followed as described previously.¹²

CASE REPORTS

Case 1

A 19-year-old Caucasian woman developed a severe, prolonged respiratory infection. Within a few weeks, the patient developed intermittent right lower quadrant abdominal pain, night sweats, nausea and moderate fatigue. Nine months after the respiratory infection, CT scan of abdomen showed circumferential thickening of the terminal ileum (figure 1A), and a colonoscopic examination of the terminal ileum showed nodular swelling with subepithelial haemorrhage (figure 1B). Pathological examination of several biopsies demonstrated lymphoid hyperplasia and chronic inflammation without granuloma; enteroviral RNA sequence was detected in the biopsy specimen by the qualitative reverse transcriptase (RT)-PCR assay. A confirmatory real-time RT-PCR assay showed approximately 100 000 copies of enteroviral RNA in an 80 μ m de-paraffinised section of five biopsies (3×2 mm).

One week after the biopsy, the patient was admitted to the hospital with severe fatigue, fevers to 102°F, night sweats, debilitating myalgia, vomiting, diarrhoea and marked leucopenia

(total absolute neutrophil count as low as 350). A bone marrow biopsy showed normal cellular elements but enteroviral RNA was detected in the paraffinised sections. IgG and IgM antibody for adenoviruses, cytomegalovirus, coxsackievirus B1-6 and echovirus 6, 7, 9, 11 and 30 were negative; human herpesvirus 6 and Epstein–Barr IgG titres were positive but respective IgM antibodies were negative. Two doses of intravenous immuno-globulin were administrated with improvement of her symptoms over the next 2 months. Leucopenia eventually resolved.

The patient continued to have debilitating fatigue, myalgia, headaches, cognitive dysfunction, sore throat, mild respiratory and GI symptoms, and mild weight loss. Two years after hospitalisation, a stomach biopsy showed extensive viral protein in the antrum (figure 1C), and enteroviral RNA sequence was detected in the same specimen. Her symptoms improved after taking Chinese herbs for few months.

Case 2

A 44-year-old white woman with known history of asymptomatic hepatitis C infection developed severe diarrhoea and vomiting in August 2002, followed by aspiration pneumonia requiring intubation and ventilator support. The alanine leucine transferase level rose to 1340 (normal <45) and hepatitis C RNA was detectable. She was discharged 10 days later but was readmitted to the hospital 2 weeks later with mental confusion, diffuse myalgia, severe cognitive function, bed-ridden fatigue and headache. MRI scan of the brain showed diffuse white matter changes; and study of the cerebrospinal fluid showed elevated IgG synthesis rate and myelin basic protein, consistent with acute disseminated encephalomyelitis. The patient became comatose on day 3 of high dose intavenous steroids, and the therapy was changed to a 2-week course of daily intravenous immunoglobulin (0.4 g/kg/day). Coxsackievirus B5 antibody was greater than 1:640; and enteroviral RNA was detected in the PBLs on two occasions prior to infusion of intravenous immunoglobulin. Hepatitis C RNA was not detected from then on.

Figure 1 (A) Thickened wall of a segment of terminal ileum by CT scan of abdomen with contrast (arrow). (B) Nodular swelling of terminal ileum with subepithelial haemorrhage, by direct colonoscopic examination. (C) Immunoperoxidase staining of stomach biopsy demonstrates extensive enterovirus protein (\times 100). (D) There was no staining with control monoclonal antibody directed against cytomegalovirus (\times 100).



Over the next 6 years, serial MRI scans showed gradual improvement of brain white matter changes; the patient had mild improvement of cognitive function, ambulation, although she remained severely fatigued with night sweats, diffuse intense myalgia, postexertional malaise, sleep disturbance, headache, nausea and diarrhoea, sore throat and cervical lymphadenopathy. Four years after the initial infection, a biopsy of stomach antrum was positive for enterovirus VP 1 protein and enterovirus RNA.

Case 3

A 40-year-old white man with a history of recurrent diarrhoea and cardiac arrhythmia 1 year earlier developed acute asthmatic bronchitis after ill contact. After he failed to respond to oral corticosteroids and antibiotics, the patient was hospitalised on three separate occasions over the next 2 months for continuing, severe respiratory symptoms, vomiting, diarrhoea, dyspnoea, myalgia and headaches; these were treated with antibiotics and higher doses of corticosteroids. Bronchial washing obtained during the third hospitalisation tested positive for enterovirus RNA, but negative for other viruses, bacteria, mycobacteria and fungus.

Enteroviral RNA was found in his PBLs twice over the next 3 months. He developed debilitating fatigue, continuing myalgia, headache, difficulty with concentration, sore throat and cervical lymphadenopathy, and insomnia for the next 4 years, along with persistently elevated echovirus 6 antibody of \geq 1:640 (normal <1:10; ARUP Laboratory, Salt Lake City, Utah, USA). He did not respond to the combination of interferon α -2a and ribavirin,¹³ but significantly improved after a 6-month treatment with a combination of pegylated interferon α -2a and interferon γ .¹¹ He was able to return to work 2 years after the hospitalisation, but energy level remained less than 60% of normal, and aforementioned symptoms continued at a lower level. A stomach biopsy performed 4 years after the onset of the respiratory illness demonstrated extensive viral protein and enterovirus RNA in the antrum, confirmed by sequencing.

DISCUSSION

All three patients developed acute enterovirus infections, documented by the presence of enteroviral RNA in the infected secretion, blood or affected tissues, and this was followed over the next few years by multitude of symptoms consistent with the diagnosis of ME/CFS.⁷ Years after the acute infections with respiratory/GI symptoms, the persistence of viral protein and RNA was demonstrated in the stomach biopsies.

All three patients had lymphopenia during the acute infection, but more extensive immunological studies were not performed at that time. We recently reported profound decline of CD8+ T lymphocytes, and to a lesser extent CD4+ lymphocytes, in patients with acute enterovirus infection associated with reactivation of varicella-zoster virus, although the lymphocyte count returned to normal after 2 months.¹⁴ An immunosuppressive effect of the acute enteroviral infection was likely to have predisposed to viral persistence in some of the patients.

These case histories illustrate several important clinical observations. Enteroviruses acquired through the respiratory route were swallowed into the GI tract. As we have seen in patient 1 and other patients with the initial respiratory infections, the GI symptoms often started within one to a few weeks. It is plausible that the initial inflammatory response in the respiratory tract prevented viral replication in the GI tract; and the latter infection would only manifest after cessation of the immune response against the first round of viral infection.

In patient 1, circumferential thickening of terminal ileum demonstrated by CT scan and the nodularity observed by direct visualisation was consistent with swelling of the Peyer's patches, a known site of enterovirus replication,¹⁵ but the pathological examination of biopsies showed no granuloma, cytopathic changes or inclusions body. The underlying infection would have been missed had enterovirus RNA not been searched for in the biopsies. The high level of enterovirus RNA found in the Peyer's patches 9 months after the initial respiratory and GI symptoms suggested a persistent infection, and was most likely to have explained the severe relapse the patient experienced 1 week after the colonoscopy and biopsies. During acute exacerbation of viral infection associated with severe leucopenia, enterovirus RNA was also found in the bone marrow, another site of viral dissemination, as reported previously.¹¹ To our knowledge, persistence of viral genome has not been previously demonstrated in bone marrow and terminal ileum of immunocompetent patients. Even though the symptoms of acute viral exacerbation and leucopenia improved after intravenous immunoglobulin treatment, the patient had persistent symptoms of ME/CFS afterwards, with documented viral persistence in the stomach years later.

Prior infection could change the immune response to the next viral insult, as has been demonstrated in animal model.^{16 17} Preexisting hepatitis C in patient 2, and the chronic, recurrent diarrhoea in patient 3 could have polarised the immune system toward an aberrant and ineffective response favouring the development of viral persistence.¹⁸ Interestingly, the immuno-logical response developed in patient 2 eradicated the low-grade hepatitis C infection but was unable to eliminate enteroviruses from her tissues.

Persistent virus genome has been demonstrated in the pancreases, muscles and hearts of animal and human patients with type 1 diabetes, viral myocarditis and viral myositis, respectively.^{4 5} ^{19–24} Our study demonstrated enteroviral protein, viral RNA and non-cytopathic viruses in the stomach tissues taken from patients with ME/CFS.¹² A recent study on poliovirus trafficking in mice after oral inoculation of poliovirus quasispecies not only demonstrated replication between viruses isolated from the stomach and brain.²⁵ The correlation between the blood quasispecies and the stomach or brain isolates was quite low. This observation lends support to further study the viruses isolated from the stomach, which could serve as a surrogate site for the brain or other organs where human tissues could not be easily obtained.

One has to address Koch's postulate when considering the causality of an infectious agent. Enteroviruses are well-known causes of acute, localised and systemic infections.¹ Enterovirus has been repeatedly isolated from the cerebrospinal fluid of patients with agammaglobulinaemia, and this confirmed the concept of chronic persistence in immunocompromised host.² In patients with ME/CFS, we demonstrated transient growth of non-cytopathic enteroviruses from stomach biopsies many years after initial infections. To date, culturing experiments of these slow-growing, non-cytopathic viruses and characterisation are ongoing, but the infectivity in a suitable animal model will likely be quite low, if possible at all. Perhaps, it is more appropriate to consider the issue of virulence versus attenuation of an infectious organism, as Koch's and Pasteur's views and research differed on anthrax. Koch believed that the biological and chemical characteristics of a microbial species were not only specific but also permanent, which contradicted Pasteur's concept that microbial virulence is not constant but, instead, is a variable property of microbial species-a property that can be lost but also

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Take-home messages

- Myalgia encephalomyelitis/chronic fatigue syndrome (ME/ CFS) can follow acute enterovirus infection with documented persistent infection of the stomach.
- Familiarity with the protean manifestations of acute enterovirus infections and the diagnostic tests will help identify specific aetiology in patients before the development of ME/CFS.
- Persistent respiratory and/or abdominal complaints following acute viral infections are indicative of persistent enterovirus infection, and viral persistence can be demonstrated by viral protein staining of the stomach biopsies.
- Chronic enterovirus infection in an immunocompetent host may be an example of a stalemate between attenuated, intracellular viruses and an ineffective immune response.

recovered.²⁶ For protection against the natural infection, and in itself unlikely to cause the disease, an infectious organism could be attenuated for the purpose of vaccination. An organism capable of chronic infection without causing significant fatality is unlikely to virulent but rather more attenuated to persist in the host. Chronic enterovirus infection in an immunocompetent host may be an example of a stalemate between attenuated, intracellular viruses and an ineffective immune response; the latter may be related to the initial immunosuppression induced by the acute infection. Whether the defective viruses we found in the stomach biopsies could revert back to full virulence under severe immunosuppression is unknown and certainly deserves further investigations.

Since routine screening for viral infections has not been a common practice, most of the cases of enterovirus infections are not properly diagnosed and the proportion of patients developing chronic symptoms after acute enterovirus infection remains unknown.^{1 27} A number of viruses can cause respiratory and GI infections, although few have been shown to cause persistent symptoms in the immunocompetent patients.^{8–11} A Luminex multiplex test, xTAG(TM) Respiratory Viral Panel, for 18 respiratory viruses has just become available in North America.²⁸ A more accurate of diagnosis of acute viral infections may further clarify the impact of these viruses on the development of ME/CFS.

 $\label{eq:competing interests} \mbox{EV Med Research has a patent for using the immunoperoxidase test to diagnose ME/CFS.$

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