Antibody to parvovirus B19 nonstructural protein is associated with chronic arthralgia in patients with chronic fatigue syndrome/myalgic encephalomyelitis

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Chronic fatigue syndrome/myalgic encephalomyelitis (CFS/ME) is a neuro-immune disease of uncertain pathogenesis. Human parvovirus B19 infection has been shown to occur just prior to development of the onset of CFS/ME in several cases, although B19 seroprevalence studies do not show any significant differences between CFS/ME and controls. In this study, we analysed parvovirus B19 markers in CFS/ME patients (n=200), diagnosed according to Fukuda CDC criteria, and normal blood donors (n=200). Serum from each subject was tested for anti-B19 VP2 IgM and IgG (by Biotrin ELISA), anti-B19 NS1 IgM and IgG (by immunofluorescence), and B19 DNA (by real-time PCR). CFS/ME patients and normal blood donors had a similar B19 seroprevalence (75 % versus 78 %, respectively). Eighty-three CFS patients (41.5 %) as compared with fourteen (7 %) normal blood donors tested positive for anti-B19 NS1 IgG ($\chi^2=64.8; P<0.0001$; odds ratio=9.42, CI 5.11–17.38). Of these 83 patients, 61 complained of chronic joint pain, while 22 did not. Parvovirus B19 DNA was detected in serum of 11 CFS patients and none of the controls by Taqman real-time PCR ($\chi^2=9.35, P<0.002$). Positivity for anti-B19 NS1 IgG was associated with higher expression levels of the human CFS-associated genes NHLH1 and GABPA. As NS1 antibodies are thought to indicate chronic or severe courses of B19 infection, these findings suggest that although the seroprevalence of B19 in CFS patients is similar to controls, the immune control of the virus in these patients may not be efficient.

INTRODUCTION

Chronic fatigue syndrome/myalgic encephalomyelitis (CFS/ME) is a disease characterized by severe and debilitating fatigue, sleep abnormalities, impaired memory and concentration, and musculoskeletal pain (Fukuda et al., 1994). In the Western world, the population prevalence is estimated to be of the order of 0.5 % (Report of the CFS/ME Working Group, 2002; Papanicolaou et al., 2004). Several factors have been proposed to account for the pathogenesis of CFS; however, the most widely believed theory is that the symptoms of this disease are mediated by immune mechanisms which, in turn, may be somehow induced by virus infection (Komaroff & Buchwald, 1998; Devanur & Kerr, 2006).

Human parvovirus B19 (B19) is the aetiological agent of the rash illness erythema infectiosum, arthralgia and arthritis, transient aplastic crisis in patients with shortened red cell survival, fetal death and pure red cell aplasia in immunocompromised persons (Kerr & Modrow, 2006). B19 infection has also been shown to occur just prior to the onset of symptoms of CFS in several case reports and follow-up studies (Kerr et al., 2002). However, B19 seroprevalence in CFS patients is similar to that in the general population.
The B19 genome consists of a linear, single-stranded DNA of ~5.6 kb, encoding the two capsid proteins VP1 and VP2 from the right side and the non-structural protein (NS1) from the left side (Astell et al., 1997). The NS1 protein possesses DNA-nicking and helicase activities, is cytotoxic and trans-activates the human interleukin-6 gene (Moffatt et al., 1996). Immunity to B19 virus is mediated by both humoral and cellular means. Antibodies to NS1 occur in approximately 30% of infected individuals and may indicate more persistent and severe courses of B19 infection (Searle et al., 1998).

The present study was undertaken in view of the paucity of knowledge of the role of B19 infection in CFS patients and the human immune response to it. Findings of this study confirm that B19 seroprevalence in CFS patients is similar to that in the normal population. However, antibodies to the NS1 protein were elevated in frequency in CFS patients and were associated with both chronic arthralgia and higher expression levels of the human CFS-associated genes NHLH1 and GABPA.

**RESULTS**

**Subjects and clinical characterization**

A total of 200 CFS/ME patients fulfilling Fukuda CDC diagnostic criteria and 200 normal healthy blood donors of equivalent age and gender were included in this study. A summary of the clinical details of these CFS patients is shown in Table 1. There were obvious differences between the two groups with regard to the frequency and severity of CFS-associated symptoms, and of scores on clinical questionnaires (Table 1), as would be expected.

**Parvovirus B19 markers**

The CFS/ME patients and normal blood donors had a similar B19 seroprevalence (anti-B19 VP2 IgG) (75% versus 78%, respectively). Four CFS patients were found to be positive for anti-B19 VP2 IgM; however, these patients reported no symptoms which would suggest acute B19 infection against a background of CFS. Of these four IgM-positive patients, two were positive for B19 DNA by PCR; however, none was positive for either anti-B19 VP2 IgG or anti-B19 NS1 IgM. Eighty-three CFS patients (41.5%) as compared with fourteen (7%) normal blood donors tested positive for anti-B19 NS1 IgG ($\chi^2 = 64.8; P < 0.0001$; odds ratio = 9.42, CI 5.11–17.38). Of these 83 patients, 61 complained of chronic joint pain, while 22 did not. Three CFS patients were positive for anti-B19 NS1 IgM, as compared with one of the controls. Parvovirus B19 DNA was detected in serum of 11 CFS patients, but none of the normal blood donors, by Taqman real-time PCR; 5 of these 11 were positive for anti-NS1 IgG antibody. The B19 DNA viral load in positive samples ranged from $10^4$ to $10^3$ genome copies per ml blood.

**Human CFS-associated genes**

The fold-differences of expression between CFS and normal groups for the human CFS-associated genes NHLH1 and GABPA were 13.5 and 7, respectively, results which confirm our previous findings (Kerr et al., 2008; Zhang et al., 2010). Although in most CFS patients these genes were raised above the mean level found in normal persons, we observed significant heterogeneity in expression within the CFS group, with some patients exhibiting very high levels and others exhibiting lower levels. The expression levels of the two genes were strongly associated ($P = 0.0012$) with each other in all subjects, regardless of whether they were CFS patients or normal blood donors; this association was statistically significant ($P = 0.0012$).

In CFS patients, positivity for anti-B19 NS1 IgG was associated with higher expression levels of both NHLH1 (fold difference 2.4; $P = 0.006$) and GABPA (fold difference 1.6; $P = 0.003$). However, this association was not seen in the normal controls.

**DISCUSSION**

This study was undertaken to clarify an apparent paradox: acute parvovirus B19 infection may somehow induce development of CFS in a subset of patients, yet patients with CFS exhibit similar B19 seroprevalence to the normal population. This study confirms that CFS patients have similar B19 seroprevalence to normal healthy controls. However, we found that CFS patients have a much higher prevalence of anti-B19 NS1 antibody.

The major B19 nonstructural protein, NS1 (77 kDa), is a multifunctional protein. It has been shown to possess site-specific DNA-binding and helicase activities, and is functionally active as a trans-activator of the viral p6 and various cellular promoters (Gareus et al., 1998; Raab et al., 2001, 2002; Vassias et al., 1998), for example, the cellular promoters for the expression of TNF-α and interleukin 6 (Fu et al., 2002; Moffatt et al., 1996). NS1 also contains a well-conserved nucleoside-triphosphate-binding motif, which is essential for a variety of biological functions, such as ATPase activity and cytotoxicity.

In immunocompetent patients with persistent parvovirus B19 infections, VP1- and VP2-specific IgG antibodies are detected in combination with NS1-specific antibodies (von Poblotzki et al., 1995a, b; Hemauer et al., 1999, 2000; Kerr & Cuniffe, 2000; Lehmann et al., 2002). Usually, the synthesis of these NS1 antibodies follows anti-VP1/VP2-IgG and starts at about 3–4 weeks after infection (Hemauer et al., 2000; von Poblotzki et al., 1995a). NS1 antibodies have been reported to occur in more severe and persistent courses of B19 infection (von Poblotzki et al., 1995a, b) and one study reports their association with chronic but not acute arthritis attributable to B19 infection (Kerr & Cuniffe, 2000). Therefore, their presence in 41.5% of CFS patients as compared with 7% of normal controls in the
The present study may indicate a more severe or persistent course of B19 infection in the context of CFS, whether or not B19 virus infection was the inducing event for CFS. CFS is recognized to be an inflammatory disease which can persist for many years. Our findings are consistent with a previous study which reported that 73% of NS1 antibody-positive CFS patients suffered arthralgia (Kerr & Cunniffe, 2000). Of the 83 patients with antibodies to B19 NS1, 61 (73%) suffered from chronic arthralgia. This is similar to the results of previous studies of B19-infected patients (Kerr & Cunniffe, 2000). This is very interesting and quite unexpected.

Parvovirus B19 DNAemia was documented in 11 CFS patients as compared with none of the controls. In a previous study (Kerr et al., 2001), 4 of 5 patients with B19-associated CFS (i.e. patients followed from the time of detection of anti-B19 IgM whose CFS-like symptom onset occurred contemporaneously with detection of anti-B19 IgM, and whose symptoms persisted such that they later fulfilled diagnostic criteria for CFS) exhibited B19 DNAemia at follow-up. Therefore, this finding may suggest that the disease in these 11 patients may have been somehow induced by acute B19 infection. Such patients have previously been shown to respond very well to intravenous immunoglobulin (IVIG), the only specific treatment for parvovirus B19 infection (Kerr et al., 2003). In patients with antibodies to anti-B19 NS1, but without parvovirus B19 DNAemia, it is possible that the parvovirus infection was latent and reactivated at a low level.

The CFS-associated human genes \textit{NHLH1} and \textit{GABPA} were chosen for testing in the present study, as they were the transcription factors found to have over-representation of binding sites in most upregulated genes in a previous study of gene expression in CFS (Kerr et al., 2008). The helix–loop–helix (HLH) proteins are a family of putative transcription factors, some of which have been shown to play an important role in growth and development of a wide variety of tissues and species. The GABP complex contributes to the transcriptional regulation of a number of subunits of mitochondrial enzymes, including cytochrome \textit{c} oxidase and mitochondrial transcription factor A. The association between expression levels of \textit{NHLH1} and \textit{GABPA} is intriguing, but unexplained. Further work is required to explain this relationship.

The relationship between NS1 antibody positivity and higher expression levels of \textit{NHLH1} and \textit{GABPA} is also unexplained. Following the acute phase of infection, B19 virus DNA persists, possibly life-long in many tissues of the human body. It is possible that chronic B19 antigen

<table>
<thead>
<tr>
<th>Clinical parameter</th>
<th>CFS/ME patients ((n=200))</th>
<th>Normal blood donors ((n=200))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (M:F)</td>
<td>1:3</td>
<td>1:3</td>
</tr>
<tr>
<td>Mean age (years)</td>
<td>38.4</td>
<td>40.9</td>
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<tr>
<td>Mean duration of disease (years : months)</td>
<td>3.67</td>
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<tr>
<td><strong>Symptoms/signs</strong> (no. of subjects with each symptom)</td>
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<td></td>
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<tr>
<td>Headache</td>
<td>103</td>
<td>10</td>
</tr>
<tr>
<td>Sore throat</td>
<td>109</td>
<td>8</td>
</tr>
<tr>
<td>Poor memory/concentration</td>
<td>110</td>
<td>11</td>
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<tr>
<td>Muscle pain</td>
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<td>13</td>
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<tr>
<td>Muscle weakness</td>
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<td>Joint pain</td>
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<td>15</td>
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<td>Sleep problem</td>
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<td>Gastrointestinal problems</td>
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<td>Fainting/dizziness</td>
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<td>2</td>
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<td>Tender lymphadenopathy</td>
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<tr>
<td><strong>Mean scores</strong></td>
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<tr>
<td>Physical fatigue (Chalder)</td>
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<tr>
<td>Mental fatigue (Chalder)</td>
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<tr>
<td>McGill Pain Questionnaire</td>
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<td>Sphere Questionnaire</td>
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<td>SF-36 Questionnaire</td>
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<td>88.37</td>
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<tr>
<td>Pittsburgh Sleep Quality Index</td>
<td>11.81</td>
<td>5.14</td>
</tr>
</tbody>
</table>

NA, Not applicable.

Table 1. Patient information including age, sex, symptoms and questionnaire results summarizing fatigue severity, pain, sleep, general function, and associated symptoms for CFS/ME patients and normal blood donors.
stimulation may be responsible for an inflammatory state which could increase levels of these transcription factors, although this requires confirmation.

In conclusion, we report that the prevalence of anti-NS1 antibody is significantly increased in CFS patients and is associated with chronic joint pain and elevated levels of the CFS-associated human genes, NHLH1 and GABPA. Further work is required to determine how B19 virus could upregulate these genes in the context of CFS.

METHODS

Subject enrolment, clinical characterization and blood sampling. CFS/ME patients (n=200) from three different geographical areas in the UK and USA (New York and Boston) were diagnosed according to Fukuda diagnostic criteria for CFS/ME (Fukuda et al., 1994) and enrolled into the study. Patients with psychiatric disease were excluded from the CFS/ME group using the Minnesota International Neuropsychiatric Interview (MINI), thus ensuring that none of our CFS/ME patients was suffering from major psychiatric disease or abuse of alcohol or other drugs.

Healthy normal blood donors enrolled from the Dorset National Blood Service (NBS) (n=200) were used as a comparison group. Restrictions imposed by the NBS on those allowed to donate blood are outlined elsewhere (Kaushik et al., 2005).

For both CFS and normal groups, individuals who smoked in the previous year, who abused alcohol or other drugs, were currently taking (or were within 3 months of taking) antibiotics, steroids, cytotoxic drugs or antidepressants were excluded from the study. The only exception to this was that these criteria were not formally applied to the Boston patients (n=15); however, we believe that these patients also fulfilled these same criteria.

For all enrolled subjects (patients and controls), according to the recommendations of the International CFS Study Group (Reeves et al., 2003), severity of physical and mental fatigue was assessed using the Chalder Fatigue Scale (Chalder et al., 1993); level of disability was assessed using the Medical Outcomes Survey Short Form-36 (SF-36); accompanying symptoms were characterized using the Somatic and Psychological Health Report (SPHERE); sleep abnormalities were assessed using the Pittsburgh Sleep Questionnaire; and assessment of type and severity of pain was performed using the McGill Pain Questionnaire.

Patients and controls gave informed written consent according to guidance of the Wandsworth Research Ethics Committee (approval number 05/Q0803/137). For the New York and Boston patients, approval of the local Institutional Review Board was obtained. The human experimentation guidelines of the US Department of Health and Human Services were followed in this study.

Fifteen millilitres of blood was taken from both CFS/ME patients and normal blood donors (as part of routine blood donation) and used to inoculate PAXgene tubes (PreAnalytix), EDTA blood tubes and clotted blood tubes, for extraction of total RNA, genomic DNA and serum, respectively. Total RNA was extracted using the PAXgene blood RNA kit (PreAnalytix), according to the instructions of the manufacturer. RNA quality and amount were confirmed by microspectrophotometry (Nanodrop). Total RNA samples used in this study had an $A_{260}/A_{280}$ ratio of 1.9–2.0. cDNA was prepared from total RNA using random hexamers. Genomic DNA was extracted from EDTA blood using the DNA Blood mini kit (Qiagen). All samples were stored at $-80^\circ$C until the time of the study.

Detection of parvovirus B19 anti-VP2 antibodies. Anti-B19 VP2 IgM and IgG were detected by ELISA (Biotrin) according to the instructions of the manufacturer.

Detection of parvovirus B19 anti-NS1 antibodies. Anti-B19 NS1 antibodies were detected by immunofluorescence using SP9 cells infected with a recombinant Autographica california nuclear polyhedrosis virus (rAcNPV) with the Stu NS1 nucleotide sequence inserted just downstream of the polyhedrin promoter; this recombinant virus was kindly provided by B. J. Cohen (Virus Reference Division, Central Public Health Laboratory, Public Health Laboratory Service, Colindale, London, UK) (Hicks et al., 1996). rAcNPV-infected SP9 cells were spotted onto immunofluorescence slide wells and used for the IF assay as a source of NS1 antigen. Human anti-NS1 positive control serum from a pregnant woman with B19 infection and documented anti-NS1 antibodies was kindly provided by S. Modrow (Institut für Medizinische Mikrobiologie und Hygiene, Universität Regensburg, Regensburg, Germany).

Serum samples were diluted 1:100 in sterile PBS and used to stain both infected and uninfected SP9 cells for 30 min at 37°C in a humidified atmosphere. After thorough washing, goat anti-human IgG FITC conjugate (Sigma) was added at its working dilution of 1:32 for 30 min at 37°C. After further washing, slides were mounted and viewed on a fluorescence microscope with a filter of emission waveband at 515 nm (Leitz, Switzerland). Positive control anti-NS1 serum and PBS (negative control) were used throughout. Sera showing the characteristic pattern of positive fluorescence in infected but not uninfected cells were designated positive.

Detection of parvovirus B19 DNA. Parvovirus B19 DNA was detected by real-time PCR using primers specific to highly conserved sequences within the structural and non-structural genes, respectively, as previously described (Kerr et al., 2005). The sensitivity of both of these PCR tests was five genome copies.

Quantification of CFS-associated human genes. QPCR (Applied Biosystems) was used to quantify the amount of mRNA for the CFS/ME-associated human genes NHLH1 and GABPA by the comparative method, using custom 384-well low-density arrays and the ABI PRISM 7900HT instrument (Applied Biosystems), with GAPDH (encoding glyceraldehyde-3-phosphate dehydrogenase) as the endogenous control gene. Experiments were performed in triplicate using the protocol described previously (Kaushik et al., 2005; Kerr et al., 2008). Data were displayed using SDS 2.2 software (ABI), discordant data between replicates omitted, and results calculated. The threshold cycle ($C_t$) for each test gene in each sample was compared to that for GAPDH to calculate a $\Delta C_t$ value. $\Delta C_t$ values were then normalized to the calibrator sample to give the $\Delta \Delta C_t$ values. Relative quantities (RQ) (2$^{-\Delta \Delta C_t}$) of each mRNA were then calculated. Samples showing a difference between minimum and maximum RQ values of $\geq 100$ (indicating poor replicate concordance) were excluded. The t-test was used to compare mean RQ values between groups. A P-value of $\leq 0.05$ was taken to be significant.

Statistical testing. The $\chi^2$ test was used to test the significance of associations of B19 markers in CFS/ME patients and controls, CFS-associated symptoms and CFS-associated human genes. The t-test was used to determine the significance of association between expression levels of the two CFS-associated human genes, NHLH1 and GABPA.

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