tissue repository, which contains samples from well-characterized cohorts of CFS patients, we isolated nucleic acids from PBMCs and assayed the samples for XMRV *gag* sequences by nested polymerase chain reaction (PCR) (*5*, *6*). Of the 101 CFS samples analyzed, 68 (67%) contained XMRV *gag* sequence. Detection of XMRV was confirmed in 7 of 11 WPI CFS samples at the Cleveland Clinic by PCR-amplifying and sequencing segments of XMRV *env* [352 nucleotides (nt)] and *gag* (736 nt) in CFS PBMC DNA (Fig. 1A) (*6*). In contrast, XMRV *gag* sequences were detected in 8 of 218 (3.7%) PBMC DNA specimens from healthy individuals. Of the 11 healthy control DNA samples analyzed by PCR for both *env* and *gag*, only one sample was positive for *gag* and none for *env* (Fig. 1B). In all positive cases, the XMRV *gag* and *env* sequences were more than 99% similar to those previously reported for prostate tumor–associated strains of XMRV (VP62, VP35, and VP42) (fig. S1) (*5*).

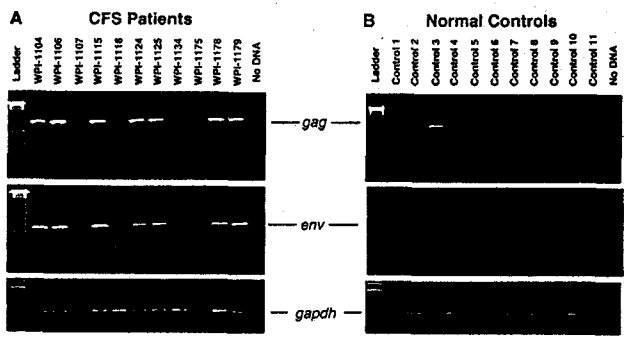

Fig. 1. XMRV sequences in PBMC DNA from CFS patients. Single-round PCR results for *gag*, *env*, and *gapdh* sequences in PBMCs of (**A**) CFS patients and (**B**) healthy controls are shown. The positions of the amplicons are indicated and DNA markers (ladder) are shown. These are representative results from one group of 20 patients.

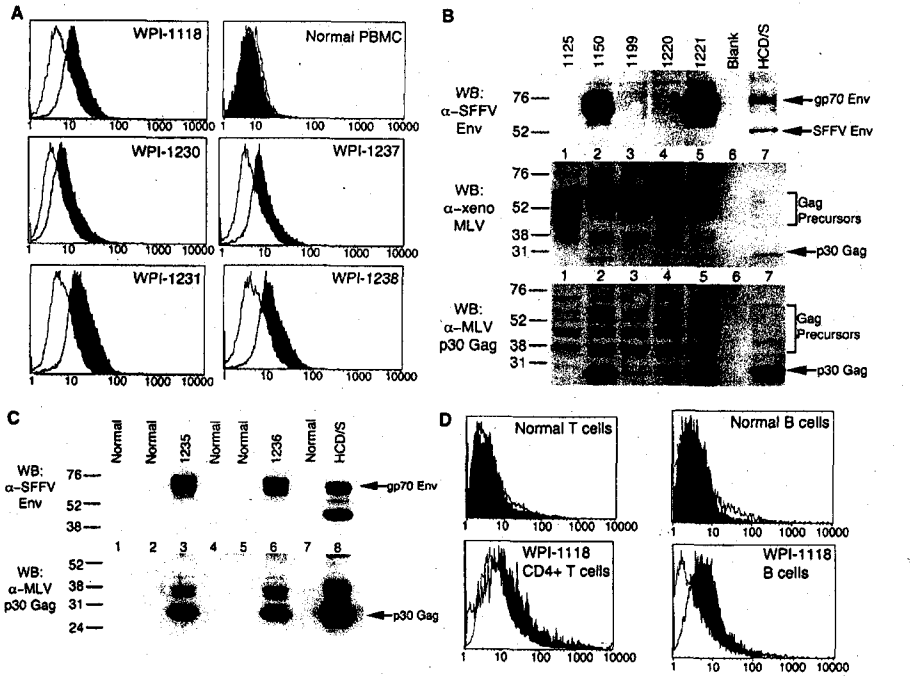

Fig. 2. Expression of XMRV proteins in PBMCs from CFS patients. (**A**) PBMCs were activated with phytohemagglutinin and interleukin-2, reacted with a mAb to MLV p30 Gag, and analyzed by IFC. (**B**) Lysates of activated PBMCs from CFS patients (lanes 1 to 5) were analyzed by Western blots with rat mAb to SFFV Env (top panel), goat antiserum to xenotropic MLV (middle panel), or goat antiserum to MLV p30 Gag (bottom panel). Lane 7, lysate from SFFV-infected HCD-57 cells. Molecular weight markers in kilodaltons are at left. (**C**) Lysates of activated PBMCs from healthy donors (lanes 1, 2, 4, 5, and 7) or from CFS patients (lanes 3 and 6) were analyzed by Western blots using rat mAb to SFFV Env (top panel) or goat antiserum to MLV p30 Gag (bottom panel). Lane 8, SFFV-infected HCD-57 cells. Molecular weight (MW) markers in kilodaltons are at left. (**D**) $CD4^+$ T cells (left) or $CD19^+$ B cells (right) were purified, activated, and examined by flow cytometry for XMRV Gag with a mAb to MLV p30 Gag.

Sequences of full-length XMRV genomes from two CFS patients and a partial genome from a third patient were generated (table S1). CFS XMRV strains 1106 and 1178 each differed by 6 nt from the reference prostate cancer strain XMRV VP62 (EF185282), and with the exception of 1 nt, the variant nucleotides mapped to different locations within the XMRV genome, suggesting independent infections. In comparison, prostate cancer–derived XMRV strains VP35 and VP42 differed from VP62 by 13 and 10 nt, respectively. Thus, the complete XMRV genomes in these CFS patients

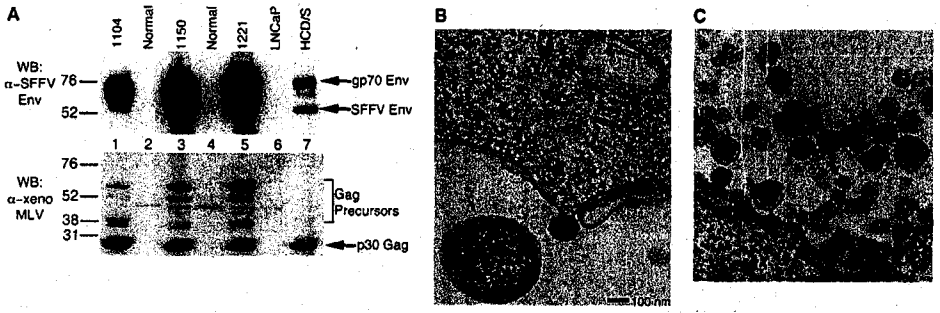

Fig. 3. Infectious XMRV in PBMCs from CFS patients. (**A**) Lysates of LNCaP cells cocultured with PBMCs from CFS patients (lanes 1, 3, and 5) or healthy donors (lanes 2 and 4) were analyzed by Western blots with rat mAb to SFFV Env (top panel) or goat antiserum to xenotropic MLV (bottom panel). Lane 6, uninfected LNCaP; lane 7, SFFV-infected HCD-57 cells. MW markers in kilodaltons are at left. (**B**) Transmission electron micrograph of a budding viral particle from LNCaP cells infected by incubation with an activated T cell culture from a CFS patient. (**C**) Transmission electron micrograph of virus particles released by infected LNCaP cells.

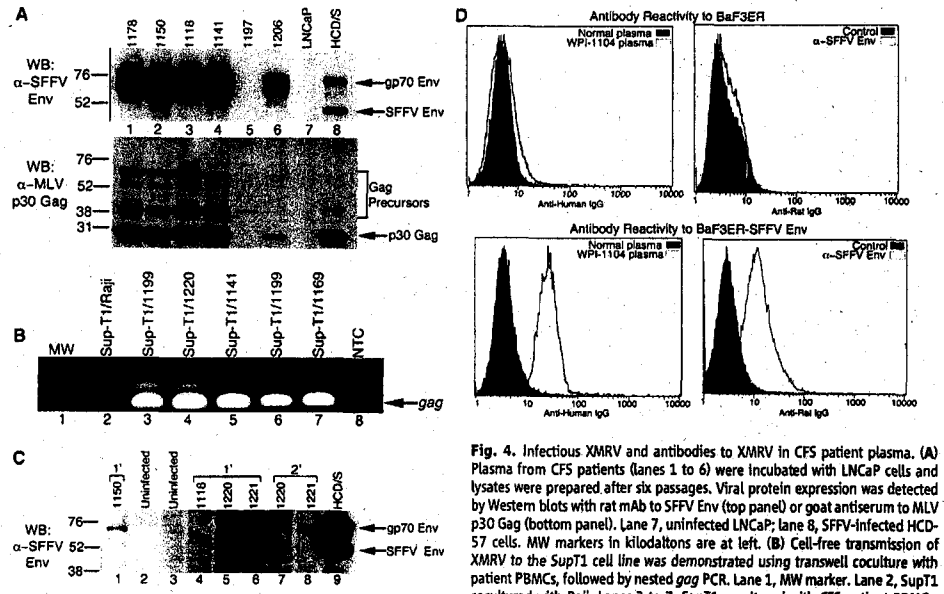

Fig. 4. Infectious XMRV and antibodies to XMRV in CFS patient plasma. (**A**) Plasma from CFS patients (lanes 1 to 6) were incubated with LNCaP cells and lysates were prepared after six passages. Viral protein expression was detected by Western blots with rat mAb to SFFV Env (top panel) or goat antiserum to MLV p30 Gag (bottom panel). Lane 7, uninfected LNCaP; lane 8, SFFV-infected HCD-57 cells. MW markers in kilodaltons are at left. (**B**) Cell-free transmission of XMRV to the SupT1 cell line was demonstrated using transwell coculture with patient PBMCs, followed by nested *gag* PCR. Lane 1, MW marker. Lane 2, SupT1 cocultured with Raji. Lanes 3 to 7, SupT1 cocultured with CFS patient PBMCs. Lane 8, no template control (NTC). (**C**) Normal T cells were exposed to cell-free supernatants obtained from T cells (lanes 1, 5, and 6) or B cells (lane 4) from CFS patients. Lanes 7 and 8 are secondary infections of normal activated T cells. Initially, uninfected primary T cells were exposed to supernatants from PBMCs of patients WPI-1220 (lane 7) and WPI-1221 (lane 8). Lanes 2 and 3, uninfected T cells; lane 9, SFFV-infected HCD-57 cells. Viral protein expression was detected by Western blot with a rat mAb to SFFV Env. MW markers in kilodaltons are at left. (**D**) Plasma samples from a CFS patient or from a healthy control as well as SFFV Env mAb or control were reacted with BaF3ER cells (top) or BaF3ER cells expressing recombinant SFFV Env (bottom) and analyzed by flow cytometry. IgG, immunoglobulin G.

were >99% identical in sequence to those detected in patients with prostate cancer. To exclude the possibility that we were detecting a murine leukemia virus (MLV) laboratory contaminant, we determined the phylogenetic relationship among endogenous (non-ecotropic) MLV sequences, XMRV sequences, and sequences from CFS patients 1104, 1106, and 1178 (fig. S2). XMRV sequences from the CFS patients clustered with the XMRV sequences from prostate cancer cases and formed a branch distinct from non-ecotropic MLVs common in inbred mouse strains. Thus, the virus detected in the CFS patients' blood samples is unlikely to be a contaminant.

To determine whether XMRV proteins were expressed in PBMCs from CFS patients, we developed intracellular flow cytometry (IFC) and Western blot assays, using antibodies (Abs) with novel viral specificities. These antibodies included, among others, (i) rat monoclonal antibody (mAb) to the spleen focus-forming virus (SFFV) envelope (Env), which reacts with all polytropic and xenotropic MLVs (*7*); (ii) goat antisera to whole mouse NZB xenotropic MLV; and (iii) a rat mAb to MLV p30 Gag (*8*). All of these Abs detected the human VP62 XMRV strain grown in human Raji, LNCaP, and Sup-T1 cells (fig. S3) (*5*). IFC of activated lymphocytes (*6, 9*) revealed that 19 of 30 PBMC samples from CFS patients reacted with the mAb to MLV p30 Gag (Fig. 2A). The majority of the 19 positive samples also reacted with antisera to other purified MLV proteins (fig. S4A). In contrast, 16 healthy control PBMC cultures tested negative (Fig. 2A and fig. S4A). These results were confirmed by Western blots (Fig. 2, B and C) (*6*) using Abs to SFFV Env, mouse xenotropic MLV, and MLV p30 Gag. Samples from five healthy donors exhibited no expression of XMRV proteins (Fig. 2C). The frequencies of CFS cases versus healthy controls that were positive and negative for XMRV sequences were used to calculate a Pearson χ^2 value of 154 (two-tailed P value of 8.1×10^{-35}). These data yield an odds ratio of 54.1 (a 95% confidence interval of 23.8 to 122), suggesting a nonrandom association with XMRV and CFS patients.

To determine which types of lymphocytes in blood express XMRV, we isolated B and T cells from one patient's PBMCs (*6*). Using mAb to MLV p30 Gag and IFC, we found that both activated T and B cells were infected with XMRV (Fig. 2D and fig. S4A). Furthermore, using mAb to SFFV Env, we found that >95% of the cells in a B cell line developed from another patient were positive for XMRV Env (fig. S4B). XMRV protein expression in CFS patient–derived activated T and B cells grown for 42 days in culture was confirmed by Western blots (fig. S4C) using Abs to SFFV Env and xenotropic MLV.

We next investigated whether the viral proteins detected in PBMCs from CFS patients represent infectious XMRV. Activated lymphocytes (*6*) were cocultured with LNCaP, a prostate cancer cell line with defects in both the JAK-STAT and RNase L pathways (*10, 11*) that was previously shown to be permissive for XMRV infection (*12*). After coculture with activated PBMCs from CFS patients, LNCaP cells expressed XMRV Env and multiple XMRV Gag proteins when analyzed by Western blot (Fig. 3A) and IFC (fig. S5A). Transmission electron microscopy (EM) of the infected LNCaP cells (Fig. 3B), as well as virus preparations from these cells (Fig. 3C), revealed 90- to 100-nm-diameter budding particles consistent with a gamma (type C) retrovirus (*13*).

We also found that XMRV could be transmitted from CFS patient plasma to LNCaP cells when we applied a virus centrifugation protocol to enhance infectivity (*6, 14, 15*). Both XMRV gp70 Env and p30 Gag were abundantly expressed in LNCaP cells incubated with plasma samples from 10 of 12 CFS patients, whereas no viral protein expression was detected in LNCaP cells incubated with plasma samples from 12 healthy donors (Fig. 4A). Likewise, LNCaP cells incubated with patient plasma tested positive for XMRV p30 Gag in IFC assays (fig. S5B). We also observed cell-free transmission of XMRV from the PBMCs of CFS patients to the T cell line SupT1 (Fig. 4B) and both primary and secondary transmission of cell-free virus from the activated T cells of CFS patients to normal T cell cultures (Fig. 4C). Together, these results suggest that both cell-associated and cell-free transmission of CFS-associated XMRV are possible.

We next investigated whether XMRV stimulates an immune response in CFS patients. For this purpose, we developed a flow cytometry assay that allowed us to detect Abs to XMRV Env by exploiting its close homology to SFFV Env (*16*). Plasma from 9 out of 18 CFS patients infected with XMRV reacted with a mouse B cell line expressing recombinant SFFV Env (BaF3ER-SFFV-Env) but not to SFFV Env negative control cells (BaF3ER), analogous to the binding of the SFFV Env mAb to these cells (Fig. 4D and S6A). In contrast, plasma from seven healthy donors did not react (Fig. 4D and fig. S6A). Furthermore, all nine positive plasma samples from CFS patients but none of the plasma samples from healthy donors blocked the binding of the SFFV Env mAb to SFFV Env on the cell surface (fig. S6B). These results are consistent with the hypothesis that CFS patients mount a specific immune response to XMRV.

Neurological maladies and immune dysfunction with inflammatory cytokine and chemokine up-regulation are some of the most commonly reported features associated with CFS. Several retroviruses, including the MLVs and the primate retroviruses HIV and HTLV-1, are associated with neurological diseases as well as cancer (*17*). Studies of retrovirus-induced neurodegeneration in rodent models have indicated that vascular and inflammatory changes mediated by cytokines and chemokines precede the neurological pathology (*18, 19*). The presence of infectious XMRV in lymphocytes may account for some of these observations of altered immune responsiveness and neurological function in CFS patients.

We have discovered a highly significant association between the XMRV retrovirus and CFS. This observation raises several important questions. Is XMRV infection a causal factor in the pathogenesis of CFS or a passenger virus in the immunosuppressed CFS patient population? What is the relationship between XMRV infection status and the presence or absence of other viruses that are often associated with CFS (e.g., herpesviruses)? Conceivably these viruses could be cofactors in pathogenesis, as is the case for HIV-mediated disease, in which co-infecting pathogens play an important role (*20*). Patients with CFS have an elevated incidence of cancer (*21*). Does XMRV infection alter the risk of cancer development in CFS? As noted above, XMRV has been detected in prostate tumors from patients expressing a specific genetic variant of the *RNASEL* gene (*5*). In contrast, in our study of this CFS cohort, we found that XMRV infection status does not correlate with the *RNASEL* genotype (*6*) (table S2).

Finally, it is worth noting that 3.7% of the healthy donors in our study tested positive for XMRV sequences. This suggests that several million Americans may be infected with a retrovirus of as yet unknown pathogenic potential.

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Note added in proof: V.C.L. is operations manager of Viral Immune Pathologies Laboratory, which is in negotiations with the Whittemore Peterson Institute to offer a diagnostic test for XMRV.

Supporting Online Material
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Materials and Methods
Figs. S1 to S6
Tables S1 and S2
References

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Complete Reconstitution of a Highly Reducing Iterative Polyketide Synthase

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Highly reducing iterative polyketide synthases are large, multifunctional enzymes that make important metabolites in fungi, such as lovastatin, a cholesterol-lowering drug from *Aspergillus terreus*. We report efficient expression of the lovastatin nonaketide synthase (LovB) from an engineered strain of *Saccharomyces cerevisiae*, as well as complete reconstitution of its catalytic function in the presence and absence of cofactors (the reduced form of nicotinamide adenine dinucleotide phosphate and *S*-adenosylmethionine) and its partner enzyme, the enoyl reductase LovC. Our results demonstrate that LovB retains correct intermediates until completion of synthesis of dihydromonacolin L, but off-loads incorrectly processed compounds as pyrones or hydrolytic products. Experiments replacing LovC with analogous MlcG from compactin biosynthesis demonstrate a gate-keeping function for this partner enzyme. This study represents a key step in the understanding of the functions and structures of this family of enzymes.

Nature uses an amazing array of enzymes to make natural products (*1*). Among these metabolites, polyketides represent a class of over 7000 known structures of which more than 20 are commercial drugs (*2*). Among the most interesting but least understood enzymes making these compounds are the highly reducing iterative polyketide synthases (HR-IPKSs) found in filamentous fungi (*3*). In contrast to the well-studied bacterial type I PKSs that operate in an assembly line fashion (*4*), HR-IPKSs are megasynthases that function iteratively by using a set of catalytic domains repeatedly in different combinations to produce structurally diverse fungal metabolites (*5*). One such metabolite is lovastatin, a cholesterol-lowering drug from *Aspergillus terreus* (*6*). This compound is a precursor to simvastatin (Zocor, Merck, Whitehouse Station, NJ), a semi-synthetic drug that had annual sales of more than $4 billion before loss of patent protection in 2006 (*7*).

Biosynthesis of lovastatin proceeds via dihydromonacolin L (acid form **1**, lactone form **2**), a product made by the HR-IPKS lovastatin nonaketide synthase (LovB), with the assistance of a separate enoyl reductase, LovC (*8*) (Fig. 1). LovB is a 335-kD protein that contains single copies of ketosynthase (KS), malonyl–coenzyme A (CoA) acyltransferase (MAT), dehydratase (DH), methyltransferase (MT), ketoreductase (KR), and acyl-carrier protein (ACP) domains, as well as a section that is homologous to the condensation (CON) domain found in nonribosomal peptide synthetases (NRPSs) (*9*). It also contains a domain that resembles an enoyl reductase (ER) but lacks that activity. LovB must catalyze ~35 reactions and use different permutations of tailoring domains after each of the eight chain-extension steps to yield the nonaketide, dihydromonacolin L (**2**). This enzyme also catalyzes a biological Diels-Alder reaction during the assembly process to form the decalin ring system (*10*). In vitro studies of LovB (*11*) have been hampered by an inability to obtain sufficient amounts of the functional purified megasynthase from either *A. terreus* or heterologous *Aspergillus* hosts. As a result, the programming that governs metabolite assembly by LovB or other HR-IPKSs is not understood. Key aspects that remain to be elucidated include (i) the catalytic and structural roles of each domain in the megasynthase, (ii) substrate specificities of the catalytic domains and their tolerance to perturbation in megasynthase functions, and (iii) factors governing the choice of different combinations of domains during each iteration of catalysis. To initiate such studies, we engineered an expression system in yeast to produce large amounts of LovB and examined the influence of cofactors and the ER partner (e.g., LovC) on product formation.

The engineered *Saccharomyces cerevisiae* strain BJ5464-NpgA, which contains a chromosomal copy of the *Aspergillus nidulans* phosphopantetheinyl (ppant) transferase gene *npgA* (*12*), was the expression host. A C-terminal hexahistidine-tagged LovB was placed under the control of the *S. cerevisiae ADH2* promoter (*13, 14*) on an episomal plasmid (YEpLovB-6His). Abundant amounts of the intact LovB could be purified from the soluble fraction to near homogeneity with a final yield of ~4.5 mg/L (fig. S1). We used mass analysis of tryptic digest fragments to verify the identity of the recombinant LovB. The ACP domain of LovB was determined to be nearly completely phosphopantetheinylated by using a ppant ejection assay with high-resolution quadrupole orthogonal acceleration–time-of-flight mass spectrometry (fig. S2). To ascertain activity of the resulting LovB and to examine the necessity for cofactors, malonyl-CoA alone was first added to the purified enzyme in buffer. Whole-cell feeding studies of doubly [^{13}C, ^{2}H]-labeled acetate to cultures of *A. terreus* showed that all three acetate hydrogens were incorporated into the acetate-derived starter units for both the nonaketide and diketide moieties in lovastatin (*15*). The purified LovB can use malonyl-CoA for both chain priming and chain elongation, loading malonate with decarboxylation to make the acetyl starter unit. Although LovB is able to prime with and elongate the chain by two further condensations with malonyl-CoA, in the absence of the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH), no ketoreduction occurs. The dominant product is lactone **3** (Fig. 2A, trace i), which forms by enolization and cyclization with off-loading of the unreduced triketide. Addition of NADPH to this system enables function of the KR domain. In this and subsequent experiments, the malonyl-CoA could be conveniently synthesized in situ by malonyl-CoA synthase (MatB) from *Rhizobium trifolii* using free malonate and CoA (*16*). With KR enabled, LovB makes penta-, hexa-, and heptaketide pyrones **4** to **6**, as well as ketones **7** and **8** (Fig. 2A, trace ii). The structures were confirmed by chemical synthesis of authentic standards, except for heptaketide **6**, which proved very unstable. However, the mass increase of 26 atomic mass units for **6** and its red shift in the ultraviolet spectrum when compared to **5** are consistent with its proposed heptaketide pyrone structure (table S3). Compounds **7** and **8** result from thioester hydrolysis of penta- and hexaketides stalling on the ACP at the β-keto stage. The resulting β-keto acids spontaneously decarboxylate to afford **7** and **8**. Formation of compounds **4** to **8** illustrates that derailment in the normal programmed steps, namely the lack of methylation due to the absence of *S*-adenosylmethionine

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Failure to Detect the Novel Retrovirus XMRV in Chronic Fatigue Syndrome

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Abstract

Background: In October 2009 it was reported that 68 of 101 patients with chronic fatigue syndrome (CFS) in the US were infected with a novel gamma retrovirus, xenotropic murine leukaemia virus-related virus (XMRV), a virus previously linked to prostate cancer. This finding, if confirmed, would have a profound effect on the understanding and treatment of an incapacitating disease affecting millions worldwide. We have investigated CFS sufferers in the UK to determine if they are carriers of XMRV.

Methodology: Patients in our CFS cohort had undergone medical screening to exclude detectable organic illness and met the CDC criteria for CFS. DNA extracted from blood samples of 186 CFS patients were screened for XMRV provirus and for the closely related murine leukaemia virus by nested PCR using specific oligonucleotide primers. To control for the integrity of the DNA, the cellular beta-globin gene was amplified. Negative controls (water) and a positive control (XMRV infectious molecular clone DNA) were included. While the beta-globin gene was amplified in all 186 samples, neither XMRV nor MLV sequences were detected.

Conclusion: XMRV or MLV sequences were not amplified from DNA originating from CFS patients in the UK. Although we found no evidence that XMRV is associated with CFS in the UK, this may be a result of population differences between North America and Europe regarding the general prevalence of XMRV infection, and might also explain the fact that two US groups found XMRV in prostate cancer tissue, while two European studies did not.

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Introduction

A recent study by Lombardi *et al.* [1] describing a gamma-retrovirus infection in 68 of 101 chronic fatigue syndrome (CFS) patients was notable not only for its claim of a new viral aetiology of a hitherto controversial disease, but also for the fact that proviral DNA could be amplified from the peripheral blood mononuclear cells (PBMC) of 3.75% (8/218) of the healthy controls. This follows an earlier claim that 1.7% (5/300) of healthy Japanese blood donors carried antibodies to the same virus [2]. The virus in question is a recently discovered retrovirus, Xenotropic Murine Leukaemia Virus (MLV)-Related Virus (XMRV).

In the original identification of XMRV in prostate cancer stromal cells, Urisman *et al.* [3] confirmed by sequence analysis that XMRV is not a laboratory contaminant, as is often the case with claims of new retroviral associations with disease. It shares >90% sequence identity in *gag* and *env* (two of the three viral structural genes) with other xenotropic MLVs.

An association between XMRV and prostate cancer was strengthened with the demonstration of XMRV protein expression in malignant epithelial cells [4]. However, these results have not been duplicated in studies conducted in Europe [5–7]. Both prostate cancer and CFS have been linked to an Arg to Gln mutation at codon 462 (R462Q) in the RNaseL gene, an interferon-induced ribonuclease [8]. On activation, RNaseL destroys single stranded cellular and viral RNA, thereby preventing viral replication, blocking protein synthesis, triggering cellular apoptosis and providing an innate anti-viral response. The two US studies are of interest, not only because this would be a further example of a virus association with cancer, but because they represent the first demonstration of a gamma-retrovirus able to infect human cells, over-riding the intrinsic immune mechanisms that were believed to protect humans from MLV infection.

The XMRV sequences derived from prostate cancer tissue are identical to those from CFS patients, but differ from xenotropic MLV sequences, endorsing a genuine cross-species transmission. However, the claim that XMRV is preferentially found in prostate tumours from patients homozygous for the R462Q variant [3] is not borne out by the second prostate cancer study to find XMRV in patients [4], nor was the genetic variant detected in CFS patients carrying XMRV [5].

The finding of Lombardi *et al.* of a 67% XMRV infection rate among CFS patients, if confirmed, would have a serious impact on understanding the pathogenesis of this complex and debilitating disease and its treatment. Therefore, it was important to determine if CFS sufferers in the UK were carriers of XMRV. We have screened DNA extracts from the blood of CFS sufferers by PCRs targeted at an XMRV-specific sequence and at a sequence conserved amongst most murine retroviruses (MRV).

Methods

Patients

All patients gave written informed consent for the use of their DNA to test aetiological theories of CFS, and the study was approved by the South London and Maudsley NHS Trust Ethics Committee. The study recruited 186 patients (62% female, age range 19–70, mean 39.6±11.3years) from consecutive referrals to the CFS clinic at King's College Hospital, London. All patients had undergone medical screening to exclude detectable organic illness, including a minimum of physical examination, urinalysis, full blood count, urea and electrolytes, thyroid function tests, liver function tests, 9 a.m. cortisol and ESR. Patients were interviewed using a semi-structured interview for CFS [9] to determine whether they met international consensus criteria for CFS. All subjects met the CDC criteria [10]; patients with the Fukuda-specified exclusionary psychiatric disorders, or somatisation disorder (as per DSM-IV), were not included. The patient set studied is a well-characterised and representative sample of CFS patients who have been described previously: all were routine clinic attendees, referred within the UK National Health Service, who had taken part in prior studies of neuroendocrine functioning [11] and/or of cognitive behaviour therapy [12]. As is typical of the patients seen in this tertiary care centre, they were markedly unwell. Few were working, and 19% were members of patient support groups for CFS/ME [12–14]. The levels of fatigue in this sample were high (mean Chalder Fatigue Scale, 26.3±5.4) [15], as were levels of disability (mean Work and Social Adjustment Scale, total score 28.2±7.2) [16]. The mean GHQ-12 score [17] was 19.7±8.1. Patients had been unwell for a median of 4.0 y (range 1–28 y). Of note was that 45% said their illness definitely related to a viral illness and 45% said it might relate to a viral illness. Overall, we conclude that this sample is typical of CFS patients seen in specialist clinical services in the UK. We also know from collaborative studies that our patients resemble those seen in other specialist CFS services in the United States and Australia [18].

PCR detection of XMRV and MLV sequences. DNA was extracted from EDTA whole blood using a standard phenol-based organic deproteinisation procedure [19]. DNA concentrations were determined by absorbance at 260 nm (A_{260}). Each sample was amplified in three nested PCRs using primers targeted to an XMRV-specific sequence, to a sequence conserved amongst most MLV and, as a control for sample addition and PCR-inhibition, to a human beta-globin (hBG) sequence (Table 1). Each first-round reaction was performed in a 25 µl volume containing 0.5 units TaqGold (Applied BioSystems, Warrington, UK), 1 x TaqGold reaction buffer (Applied BioSystems), 1.5 mM Mg^{2+}, 200 mM each dNTP, 2.5 pmol each primer to which 5 µl DNA extract or control was added. Reaction conditions were one cycle of 94°C, 8 minutes, 35 cycles of 94°C 30 seconds, 55°C 30 seconds, 72°C 30 seconds and one cycle of 72°C, 7 minutes. Second round reaction mixes were identical to the first round and the sample was a 1 µl transfer from the first round reactions. Second round reaction conditions were as for the first round over 30 cycles. PCR amplicons were visualised on a 1% agarose gel stained with ethidium bromide. Each PCR run consisted of test samples, six negative (water) and two positive controls. The positive control was a dilution of a plasmid with a full-length XMRV (isolate VP62) insert, generously gifted by Dr R. Silverman. To validate the sensitivity of the PCR, an end-point dilution of the plasmid was performed. To determine specificity of the PCR, a sample of human DNA from the LNCaP prostate cancer cell line (American Type Culture Collection, code CRL-1740) was amplified with the XMRV and MLV primer sets. To ensure integrity of the DNA extracts, three randomly selected samples were titrated to end-point using the hBG PCR to determine if the PCR copy number equated with the A_{260}. To determine if the DNA extracts exhibited low level non-specific inhibition of PCR, 10 samples were subjected to 30 cycles of the first round hBG PCR (reaction mix and conditions as above) followed by 40 cycles of a nested real-time SYBR-green PCR using the SYBR-green Fast PCR kit (Roche, Lewes UK) according to the manufacturer's instructions.

Table 1. Oligonucleotide Primers.

Target	Sequence	Location
XMRV	Forward outer 5′ CATTCTGTATCAGTTAACCTAC 3′	411–432[1]
	Reverse outer 5′ ATGATCTCGAGAACACTTAAAG 3′	606–588[1]
	Forward inner 5′ GACTTTTTGGAGTGGCTTTGT 3′	441–461[1]
	Reverse inner 5′ ACAGAAGAACAACAAAACAAATC 3′	566–544[1]
MLV	Forward outer 5′ GGATCAAGCCCCACATACAG 3′	2796–2847[2]
	Reverse outer 5′ CATCAAACAGGGTGGGACTG 3′	3179–3160[1]
	Forward inner 5′ AGAAGTCAACAAGCGGGTGG 3′	2926–2945[2]
	Reverse inner 5′ GGTGGAGTCTCAGGCAGAAA 3′	3062–3043[1]
hBG	Forward outer 5′ TGGTGGTCTACCCTTGGACC 3′	148–162[2]
	Reverse outer 5′ GAGGTTGTCCAGGTGAGCCA 3′	296–277[2]
	Forward inner 5′ GAGGTTCTTTGAGTCCTTTGG 3′	170–190[2]
	Reverse inner 5′ CATCACTAAAGGCACCGAGCA 3′	273–253[2]

Locations in GenBank accessions [1]EF185282, [2]NM000518.4.
doi:10.1371/journal.pone.0008519.t001

Results

Nested PCR Validation

Based on A_{260} of the purified plasmid, both primer sets (XMRV, MLV) were able to amplify a single target copy added to the reaction. Amplification of 600 ng of LNCaP cellular DNA added to XMRV and MLV PCRs yielded no non-specific bands when viewed on an ethidium bromide-stained agarose gel. Quantification of DNA samples from three randomly selected test samples by end-point dilution PCR with the hBG primer set showed concurrence of the PCR-determined copy number with A_{260}, thus indicating integrity of the DNA preparations. Nested real-time amplification of 10 samples showed no evidence of non-specific inhibition as determined by the slope of the amplification curves and the height of the signal plateau.

PCR Analysis of Test Samples

Input DNA ranged from 10 to 600 ng (1.6×10^3 to 1.1×10^5 cell equivalents) as determined by A_{260} of which 149 samples had an input of >100 ng and 106 samples >200 ng. None of the 186 test samples analysed yielded a specific PCR product with either the XMRV or MLV primer sets and no non-specific PCR products were observed. A specific hBG product was amplified from all 186 test samples. The positive control was amplified in each run by the

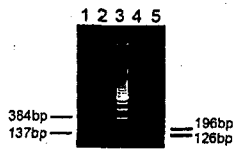

Figure 1. PCR products of the XMRV VP62 clone. Primers are generic to MLV (lanes 1 and 2) or specific to XMRV (lanes 4 and 5). The sizes of the respective fragments are shown. Lane 3–200 bp molecular size ladder.
doi:10.1371/journal.pone.0008519.g001

XMRV and MLV primer sets. A stained gel of the XMRV and MLV PCR products is shown in figure 1 and a representative sample of our results with CFS DNA and MLV primers is shown in figure 2.

Discussion

Unlike the study of Lombardi *et al.*, we have failed to detect XMRV or closely related MRV proviral DNA sequences in any sample from CFS cases. There have been numerous claims for an infective aetiology to CFS over the years, not least because, as in this sample, many patients report that their symptoms were triggered by an infective episode. Prospective epidemiological studies have confirmed that certain infective agents, for example Epstein Barr virus, are unequivocally associated with subsequent CFS [20], even if the mechanisms are unclear and almost certainly multi factorial. Nearly two decades ago, sequences from another retrovirus, the human T-lymphotropic virus type II, were amplified from the PBMCs of 10/12 (83%) adult and 13/18 paediatric CFS patients, but not from healthy control subjects [21]. However, subsequent studies carried out on small numbers (20–30) of CFS patients, failed to confirm evidence for HTLV (type I or II) [22–25] or other retroviruses, including the closely-related simian T lymphotropic virus type I, the prototype foamy virus, simian retrovirus, bovine and feline leukaemia viruses [26] and HIV-1 [23].

The Lombardi paper is the first to study a significantly larger number of people than that in any previous study and to detect a virus only recently discovered. Our study resembles that of Lombardi *et al.* in certain respects. Both studies use the widely accepted 1994 clinical case definition of CFS[10]. Lombardi *et al.* reported that their cases "presented with severe disability" and we provide quantifiable evidence confirming high levels of disability in our subjects. Our subjects were also typical of those seen in secondary and tertiary care in other centres.

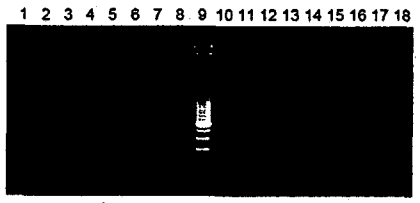

Figure 2. Nested PCR from the DNA of 8 CFS patients. Products of generic MLV primers (including XMRV) are shown. Lanes 1–8, CFS patient DNA (2nd round); lanes 9 and 10, XMRV 2nd round and 1st round positive controls; lanes 11 and 12, DNA of uninfected cell line LNCaP; lanes 13–18, water controls.
doi:10.1371/journal.pone.0008519.g002

Our own study also differs from that of Lombardi in other respects. Firstly, the PCR operator was blinded to the provenance of the DNA samples. In fact, with the exception of the PCR controls, all 186 DNA test samples originated from CFS patients. Care was taken to grow the XMRV plasmid in a laboratory in which no MLV had been cultured and no MLV vectors used and the PCR was carried out in a CPA-accredited Molecular Diagnostics Unit which processes only human tissue. Multiple (six) water (negative) controls were included in every run to detect low level contamination and a PCR to amplify a sequence that is conserved in most murine leukaemia viruses was included in order to expose any circulating MLV contamination and to detect any variant of XMRV that might be circulating in the UK CFS population.

Based on our molecular data, we do not share the conviction that XMRV may be a contributory factor in the pathogenesis of CFS, at least in the U.K.

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Author Contributions

Conceived and designed the experiments: SK MM. Performed the experiments: OWE SK. Analyzed the data: SK MM. Contributed reagents/materials/analysis tools: SK GW DC SW AC. Wrote the paper: SK MM. Facilitated the study by setting up the collaboration: JW. Responsible for providing samples and associated data from a well characterised and valuable cohort of subjects: SW.

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文献7
RETROVIROLOGY

RESEARCH Open Access

Absence of xenotropic murine leukaemia virus-related virus in UK patients with chronic fatigue syndrome

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Abstract

Background: Detection of a retrovirus, xenotropic murine leukaemia virus-related virus (XMRV), has recently been reported in 67% of patients with chronic fatigue syndrome. We have studied a total of 170 samples from chronic fatigue syndrome patients from two UK cohorts and 395 controls for evidence of XMRV infection by looking either for the presence of viral nucleic acids using quantitative PCR (limit of detection <16 viral copies) or for the presence of serological responses using a virus neutralisation assay.

Results: We have not identified XMRV DNA in any samples by PCR (0/299). Some serum samples showed XMRV neutralising activity (26/565) but only one of these positive sera came from a CFS patient. Most of the positive sera were also able to neutralise MLV particles pseudotyped with envelope proteins from other viruses, including vesicular stomatitis virus, indicating significant cross-reactivity in serological responses. Four positive samples were specific for XMRV.

Conclusions: No association between XMRV infection and CFS was observed in the samples tested, either by PCR or serological methodologies. The non-specific neutralisation observed in multiple serum samples suggests that it is unlikely that these responses were elicited by XMRV and highlights the danger of over-estimating XMRV frequency based on serological assays. In spite of this, we believe that the detection of neutralising activity that did not inhibit VSV-G pseudotyped MLV in at least four human serum samples indicates that XMRV infection may occur in the general population, although with currently uncertain outcomes.

Background

In 2006, pursuing a link between prostate cancer and an inherited mutation in the RNASEL gene, Urisman and colleagues identified a novel gammaretrovirus [1]. Using PCR methodology, this virus was shown to be present in 9/86 (10%) prostate tumours examined. It showed close sequence similarity to xenotropic murine endogenous retrovirus elements and was thus named xenotropic murine leukaemia virus related virus (XMRV). A subsequent study demonstrated receptor usage typical of murine xenotropic virus [2]. Phylogenetic analyses place XMRV firmly within the murine endogenous retroviruses [3] even though no identical element has so far been identified within the mouse genome [4]. More recently, additional groups of samples from patients with prostate cancer have been examined for the presence of XMRV with both positive [5] and negative [6,7] results.

Very recently, a paper reporting the PCR detection of XMRV in PBMC from 68/101 patients with chronic fatigue syndrome (CFS) has been published [8]. Replicating virus could be isolated from stimulated PBMC with sequences almost, but not quite identical to the viruses isolated from prostate cancer patients. Providing apparently compelling evidence against the possibility of laboratory contamination, a number of the patients were shown to have mounted an immune response against XMRV. Interestingly, around 4% of control patients appeared to harbour the virus [8].

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Replication of these results and the possible identification of roles for XMRV in the aetiology of prostate cancer and/or CFS would be of great medical significance. Detection of XMRV might provide potentially useful diagnostic tools and might also suggest therapeutic avenues for treatment. Further, widespread distribution of a potentially pathogenic virus would have important implications concerning its role as a co-factor in other conditions and in the safety of the blood supply. We therefore set out to investigate the distribution of XMRV in UK CFS patients, using PCR to search for the presence of XMRV DNA and neutralisation assays to detect an anti-XMRV immune response. In this study we did not find any association between XMRV infection and CFS.

Methods

Sample collection

Samples from the following three centres were tested; St George's University of London (SGUL), Barts and the London Hospital Trust (BLT) and Glasgow Caledonian University (GC).

The SGUL cohort comprised 142 adult CFS patients and 157 healthy blood donors. Both groups were aged between 18 and 65, and the male to female ratios were 45:97 (CFS) and 43:114 (blood donors). At the time of sampling, 2003-2008, blood was collected into three tubes (an EDTA blood tube for DNA preparation; a Paxgene tube for RNA preparation and a plain tube for serum preparation from clotted blood). CFS patients were recruited from clinics in Bristol, Dorset, London, Birmingham, Norfolk and Epsom, and all patients fulfilled diagnostic criteria of Fukuda *et al.* [9]. Blood samples were taken between 1.5 and 4 years following diagnosis. Healthy normal blood donors were enrolled from the National Blood Service (NBS), in Dorset, UK. All subjects provided informed consent, and these studies were approved by Wandsworth Research Ethics Committee, St George's Hospital, Cranmer Terrace, London SW17 0RE.

The BLT cohort comprised 226 anonymised serum samples taken in 2008-2009 (57 from the antenatal clinic; 58 with haematological disorders; 55 liver patients and 56 from the renal clinic). Clotted blood was separated by centrifugation, and the serum supernatant was removed, stored at -20°C and defrosted once. Ethical approval for the use of these samples for assay development was issued by UCLH NHS trust and adopted by chairman's action at BLT.

The GC cohort comprised 28 CFS patients (20 sera and 8 plasma samples) and 12 controls (8 sera and 4 plasma samples) from the West of Scotland catchment area. CFS patients were aged between 28 and 79, with a male to female ratio of 16:12. Samples were collected between 1995 and 2003. Controls were aged between 23 and 63, with a male to female ratio of 7:5. Samples were collected between 2002 and 2004. Some controls were relatives of the patients, and some were hospital staff volunteers. All patients met the Fukuda criteria (9). Ethical permission for blood samples to be analysed for the presence of viruses was granted by Southern General Hospital NHS Trust Local Ethics Committee.

PCR

Genomic (g)DNA was prepared from PBMC from SGUL patients and controls using the QIAamp DNA mini kit (Qiagen) and amplified using the RepliG Ultrafast Mini Kit (Qiagen), which provides highly uniform amplification of all sequences, with negligible sequence bias. The concentrations after amplification ranged from 108 - 586 ng/μl. Initially, 48 CFS patient gDNA samples were screened by single-round PCR for *gag* and *env* genes, as well as GAPDH, as outlined by Lombardi *et al.* [8] (Table 1). This PCR was performed in a 50 μl reaction volume consisting of 25 μl amplitaq gold PCR mastermix and a final DNA concentration of 2-5 ng/μl. Cycling was modified as appropriate to our mastermix; 95°C for 5 min, (95°C for 30 sec, 57°C for 30 sec, and 72°C for 60 sec) for 45 cycles, hold at 72°C for 7 min, store at 4°C. Products were visualized on 3% agarose gels by ethidium bromide staining. As we did not amplify any products using this PCR, we developed two more sensitive real-time qPCR assays which targeted 2 regions of the *env* gene, beginning at nt 6173 and

Table 1 Primer sequences used in XMRV-specific PCRs

Primer	Sequence	Reference
419F gag	ATCAGTTAACCTACCCGAGTCGGAC	Lombardi et al, 2009
1154R gag	GCCGCCTCTTCTTCATTGTTCT	Lombardi et al, 2009
5922F env	GCTAATGCTACCTCCCTCCTGG	Lombardi et al, 2009
6273R env	GGAGCCCACTGAGGAATCAAAACAGG	Lombardi et al, 2009
hGAPDH-66F	GAAGGTGAAGGTCGGAGTC	Lombardi et al, 2009
hGAPDH-291R	GAAGATGGTGATGGGATTTC	Lombardi et al, 2009
Real-time PCR		
6173 env F	GGCATACTGGAAGCCATCATCATC	
6173 env R	CCTGACCCTTAGGAGTGTTTCC	
6173 env probe	ATGGGACCTAATTTCC	
6682 env F	GTGCTGGCTGTGTCTAGTATCG	
6682 env R	GCAGAGGTATGGTTGGAGTAAGTAC	
6682 env probe	ACGGCCACCCCTTCGT	

6682, respectively (Table 1). These were used to screen samples of gDNA (prepared from PBMC) or cDNA (prepared from total RNA extracted using the Paxgene system from Preanalytix, UK) from CFS and normal blood donors. In total, 136 CFS gDNA and 140 CFS cDNA samples and 95 control gDNA and 141 control cDNA samples were analysed, such that all 142 CFS patients and 157 blood donors were screened for XMRV using these assays in either genomic DNA, cDNA or both. GAPDH was also amplified as a control using a commercial primer and probe set (Hs_99999905_m1 from Applied Biosystems). Real-time qPCR reactions were performed in 10 μl total volume, consisting of 5 μl PCR mastermix, 0.5 μl (20×) Taqman primers/probe mix, 4.5 μl sample (for gDNA, 1 μl gDNA (100-590 ng) and 3.5 μl DEPC-treated water (Ambion); for cDNA, 4.5 μl cDNA). Cycling times and temperatures were as follows. Initial denaturation occurred for 10 min at 95°C, followed by 40 cycles of denaturation at 95°C for 15 sec and combined primer annealing/extension at 60°C for 1 min. Data were displayed using SDS 1.3.1 software (ABI).

Plasmids

VP62 XMRV clone was a gift of Robert Silverman [2]. HG1 is a replication-incompetent XMRV clone constructed by site-directed mutagenesis of VP62 (the packaging signal was removed by deleting nucleotides 293-388, as numbered in GenBank EF185282; and nucleotides 7720-8108 were replaced by a BsrG1 site to remove the U3 region). Moloney-MLV Gag-Pol was expressed from KB4, a vector synthesized by cloning the *gag-pol* region from pMD-MLV GagPol [10] into pcDNA3.1. Viral genomic RNA was expressed from an MLV-based retroviral vector encoding β-galactosidase (LTR-LacZ [10]), and envelope proteins were encoded by constructs for either NZB xenotropic envelope, MLV(X) (a gift of Massimo Pizzato), Moloney-MLV env (MOSAF, a gift of Yasu Takeuchi), Friend-MLV env [10], or the G-protein from vesicular stomatitis virus (VSV-G) [11].

Virus production

Replication defective XMRV virus was prepared for neutralisation assays by co-transfecting 293T cells with HG1 and LTR-LacZ. Pseudotyped MLV was prepared by co-transfecting 293T cells with KB4, LTR-LacZ and an envelope-encoding plasmid (either MLV(X), MOSAF, Friend or VSV-G). After ~18 hours, cells were washed, and fresh media was added for a further ~24 hours, before viral supernatants were harvested, filtered, and the viral titre was measured by ELISA for RT activity (Cavidi tech). Viral stocks were titrated on D17 cells, an established, easily infectable dog cell line, or NIH-3T3 cells for Friend- and Moloney- pseudotyped MLV. After 48 hours, the cells were assayed for β-galactosidase activity using the Galacto-Star system (Applied Biosystems). The amount of virus to be used in the neutralisation assays was determined as the volume of supernatant added to 3.5×10^3 cells that resulted in ~4×10^4 counts per second of chemiluminescence.

Neutralisation assays

Neutralisation assays were performed as reported in [12]. Monoclonal antibodies to MLV Env proteins (shown in Table 2) were gifts from Leonard Evans and have been previously described [13,14]. They were provided and used as untreated hybridoma cell supernatants that were serially diluted two-fold before adding to virus to assess neutralisation activity as for serum, detailed below. Serum samples were heat inactivated at 56°C for 30 min. 5 μl serum were then added to 95 μl media in a 96-well tissue culture plate, and samples were serially diluted two-fold, leaving 50 μl at each dilution. 50 μl virus-containing supernatant were then added to each well, and the plate was incubated at 37°C for 1 hour. Following incubation, 100 μl containing 3.5×10^3 D17 cells (or NIH-3T3 cells for Friend or Moloney-MLV neutralisation) were added to each well, and the plate was incubated at 37°C. After 48 hours the cells were lysed, and β-galactosidase activity was measured. Infectivity corresponded to counts per second of chemiluminescence.

Results

PCR screening

Lombardi et al. have recently detected XMRV DNA in 67% of CFS patients by PCR [8]. To confirm an association of XMRV with this disease, we performed PCR for *gag, env* and GAPDH on 48 (of 142) CFS patient gDNA samples from SGUL using the previously published single-round PCR methodology (Table 1 and [8]. However, although all samples were positive for GAPDH, we found no evidence of XMRV DNA in any of the samples (data not shown). In case we were missing low levels of viral DNA, we devised a more sensitive qPCR-based approach. To test the sensitivity of this method, triplicate, serial 1:10 dilutions of VP62 plasmid encoding the full length XMRV genome were added to PBMC DNA from a healthy donor and tested by Taqman PCR with either env 6173 or env 6682 primers (Table 1). All replicates calculated to contain 16 copies of XMRV routinely yielded a product within 37 cycles whereas only one of three replicates of the next dilution scored positive (Figure 1). We concluded that our assay was capable of reliably detecting as little as 16 copies of proviral DNA and was therefore likely to be as sensitive, if not more so, than the assays previously used [8]. We then tested the entire SGUL panel of 142 CFS samples and 157 of the control samples (either gDNA, cDNA or both) with both env 6173 and env 6682 primers. Although positive for GAPDH, all samples were negative for XMRV. To exclude the possibility of specific sample-mediated PCR inhibition, we spiked 3 normal control cDNAs, which had previously tested negative for XMRV nucleic acid, with XMRV VP62 DNA, to a final concentration of 2.3×10^{-6} ng/μl and repeated the qPCR using both env 6173 and env 6682 primer sets. We successfully amplified the VP62 in these reactions, proving that the PCR should have amplified XMRV from the patient samples if it was present.

Table 2 Neutralisation properties of different monoclonal antibodies against XMRV and MLV pseudotyped with three different envelopes.

				Neutralisation of		
Hybridoma[1]	Raised in	Isotype	XMRV	MLV(X)	Friend	Moloney
83A25'	Rat	IgG2A	Y (88)	Y (89)	ND	ND
24-7	Mouse	IgMK	N	N	ND	ND
48	Mouse	IgG2A	N	N	Y (95)	Y (83)
538	Mouse	IgM	N	N	N	Y (63)
603	Mouse	IgM	N	Y (96)	N	ND
609	Mouse	IgM	Y (71)	N	ND	ND
610	Mouse	IgM	N	Y (64)	ND	ND
613	Mouse	IgM	N	Y (91)	ND	ND
615	Mouse	IgM	N	N	ND	ND

Y indicates neutralisation; N indicates no neutralisation; ND is not determined
The number in brackets refers to the percentage neutralisation at the least diluted antibody concentration.
[1] See references [13] and [14] for description of hybridoma cell lines

Neutralisation assays

In light of the negative data obtained using PCR assays, we set out to search for evidence of XMRV infection using a second method. Viral infection can elicit a neutralising antibody response [12]. Demonstration of such a neutralising activity can be taken as evidence for a viral infection, perhaps in cell types that were not sampled in blood. Defining neutralisation is difficult in the absence of known positive and negative sera. However, a number of neutralising monoclonal antibodies directed against the Env protein of murine retroviruses have been described [13,14]. We therefore obtained several of these (gifts of Leonard Evans) and tested them for neutralisation of XMRV and NZB xenotropic MLV (X) as well as ecotropic Friend and Moloney MLV (Table 2) by assaying for a reduction in virus infectivity following incubation of virus-containing supernatant

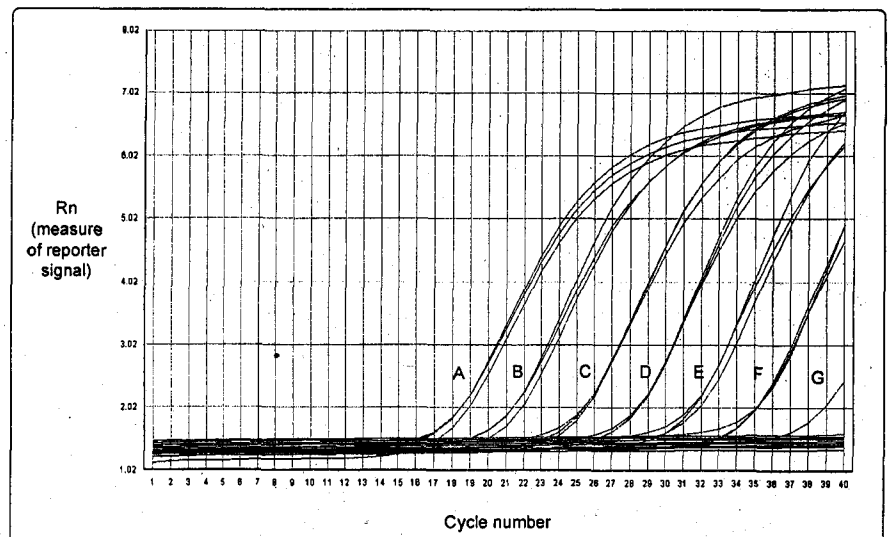

Figure 1 Sensitivity of PCR screening for XMRV in PBMC DNA. VP62 plasmid was serially diluted 1:10 into PBMC DNA from a healthy donor and tested by Taqman PCR with env 6173 primers and probe. The final amount of VP62 DNA in the reaction was A, 2.3×10^{-2} ng, B, 2.3×10^{-3} ng, C, 2.3×10^{-4} ng, D, 2.3×10^{-5} ng, E, 2.3×10^{-6} ng, F, 2.3×10^{-7} ng or G, 2.3×10^{-8} ng. The limit of sensitivity was 2.3×10^{-7} ng (shown by trace F) which equates to 16 molecules of VP62 XMRV clone.