

Detection of MLV-related virus gene sequences in blood of patients with chronic fatigue syndrome and healthy blood donors

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Chronic fatigue syndrome (CFS) is a serious systemic illness of unknown cause. A recent study identified DNA from a xenotropic murine leukemia virus-related virus (XMRV) in peripheral blood mononuclear cells (PBMCs) from 68 of 101 patients (67%) by nested PCR, as compared with 8 of 218 (3.7%) healthy controls. However, four subsequent reports failed to detect any murine leukemia virus (MLV)-related virus gene sequences in blood of CFS patients. We examined 41 PBMC-derived DNA samples from 37 patients meeting accepted diagnostic criteria for CFS and found MLV-like virus *gag* gene sequences in 32 of 37 (86.5%) compared with only 3 of 44 (6.8%) healthy volunteer blood donors. No evidence of mouse DNA contamination was detected in the PCR assay system or the clinical samples. Seven of 8 *gag*-positive patients tested again positive in a sample obtained nearly 15 y later. In contrast to the reported findings of near-genetic identity of all XMRVs, we identified a genetically diverse group of MLV-related viruses. The *gag* and *env* sequences from CFS patients were more closely related to those of polytropic mouse endogenous retroviruses than to those of XMRVs and were even less closely related to those of ecotropic MLVs. Further studies are needed to determine whether the same strong association with MLV-related viruses is found in other groups of patients with CFS, whether these viruses play a causative role in the development of CFS, and whether they represent a threat to the blood supply.

xenotropic murine leukemia virus-related virus | murine leukemia virus-like virus | viral *gag* gene sequence | polytropic | mouse mitochondria DNA PCR

Chronic fatigue syndrome (CFS) is a debilitating disorder defined solely by clinical symptoms (1) and the exclusion of other diseases; its distribution is wide and its cause is unknown. In many instances, the illness starts suddenly with an infectious-like syndrome. A number of objective immunological and neurological abnormalities have been found more often in patients with CFS than in healthy controls or in patients with other fatigue-inducing illnesses (2). Various microbial and viral infections have been implicated as possible triggers of CFS, including human herpesvirus-6, Epstein-Barr virus, enteroviruses, parvovirus B19, and the bacteria that cause Lyme disease and Q fever (2). However, no single agent has been associated with a large fraction of cases.

A recent study reported that a high percentage of patients with CFS are infected with a mouse leukemia retrovirus that has been designated xenotropic murine leukemia virus-related virus (XMRV) (3), a virus first identified in samples of human prostate cancer tissue about 4 y ago (4). However, two subsequent studies failed to find an infectious murine leukemia virus (MLV)-related virus in German prostate cancer patients (5, 6), and four recent studies from Europe and the United States have failed to detect XMRV or an MLV-related viral gene sequence in the blood of CFS patients using PCR (7–10).

In the mid-1990s, we obtained serum and whole-blood samples from CFS patients for the investigation of possible mycoplasma

infections (11). Whole-blood, peripheral blood mononuclear cell (PBMC), and plasma samples from 37 CFS patients in the mycoplasma studies were maintained in frozen storage at -80°C . Twenty-five patients were from an academic medical center and 12 were referred by community physicians. Repeat blood samples were obtained from the academic medical center patients: four samples were obtained 2 y later and similarly kept in frozen storage, eight were obtained ~ 15 y later, in 2010, and processed for XMRV/MLV-related virus testing without being frozen.

By nested PCR assays targeting the MLV-related virus *gag* gene, using both the previously described primer sets (3, 4) and an in-house-designed primer set with highly conserved sequences from different MLV-like viruses and XMRVs, we examined DNA prepared from the blood samples of these 37 CFS patients for the presence of MLV-like virus *gag* gene sequences. In addition, RNA was prepared from the deep-frozen plasma samples of these patients and analyzed by RT-PCR assay. DNA extracted from frozen PBMC samples of 44 healthy volunteer blood donors was tested in parallel.

Results

MLV-Related Viral *gag* Gene Sequences Detected in the Blood of CFS Patients.

By nested PCR assays, targeting the mouse retrovirus *gag* gene using either the previously reported PCR primer sets (first round: 419F/1154R; second round: GAG-I-F/GAG-I-R) (3, 4) or our in-house-designed PCR primer set (first round: 419F/1154R; second round: NP116/NP117) (Fig. 1), we detected a high frequency of MLV-related virus *gag* gene sequences in patients with CFS. The NP116/NP117 is an internal primer set with highly conserved sequences in different MLV-like viruses and XMRVs (Fig. S1). After the first round of nested PCR using primer set 419F/1154R, gel electrophoresis revealed positive PCR-amplified products with the predicted size of ~ 730 bp in 21 of 41 PBMC or whole-blood samples from 37 CFS patients (Fig. 1A). The nested PCR results produced by the second round of amplification using either the internal primer set GAG-I-F/GAG-I-R (with a predicted size of an ~ 410 -bp product) or the internal primer set NP116/NP117 (with a predicted size of an ~ 380 -bp product) were essentially identical. Overall, samples from 32 of 37 (86.5%) CFS patients revealed positive amplification products with the correct predicted sizes in the nested PCR (Fig. 1B). Of the 25 CFS patients who had been rigorously evaluated at the academic medical center, 24 (96%) were positive. On repeated testing 2 y

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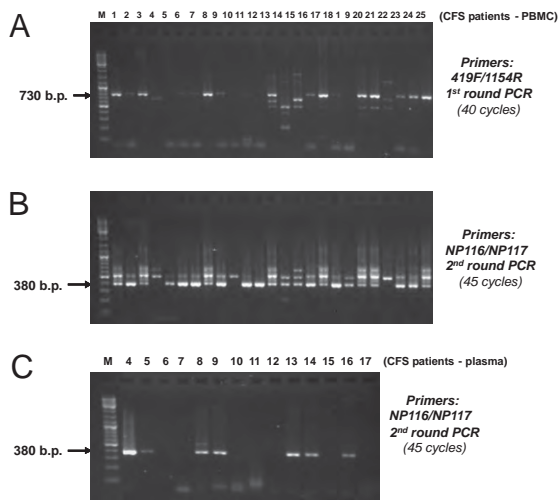


Fig. 1. MLV-related *gag* gene sequences detected in blood DNA from CFS patients. (A) Results of PBMC DNA from CFS patient samples 1–25 (of 41 samples examined) amplified after the first round of nested PCR using a previously published primer set (419F/1154R); targeting *gag* gene. (B) Results of PBMC DNA from the 25 CFS samples after completing the second round of nested PCR using an in-house–designed PCR primer set (NP116/NP117). (C) MLV-related *gag* gene RNA sequences are detected in plasma of CFS samples by RT-nested PCR. Results of RT-nested PCR for RNA derived from the plasma samples of CFS patients 4–17 are shown. The positions of expected sizes of the “positive” PCR amplicons are indicated by arrows. M, DNA ladder size markers. All positive PCR amplicons with the expected size have been confirmed by DNA sequencing.

later of four of the academic center patients, all four remained positive. On repeated testing of eight academic center patients ~15 y later (in 2010), seven remained positive. All PCR products with the correct predicted size were retrieved from the gel and analyzed by DNA sequencing. Their DNA sequences were all confirmed to be those of MLV-related virus *gag* genes. The alignments of all of the sequences obtained from PCR products of ~730 bp are shown in Fig. S1. All of the positive PCR products amplified from the CFS patients’ samples using primer set 419F/1154R were 746 bp in length. All of the positive PCR products amplified from the patients’ blood samples using primer set GAG-I-F/GAG-I-R and primer set NP116/NP117 were 413 and 380 bp in length, respectively.

In 42% of samples, we also detected and sequence-confirmed the presence of MLV-related viral RNA in the frozen plasma samples of these CFS patients, using an RT-PCR assay (Fig. 1C). With one exception, all of the patients who tested positive for viral RNA *gag* gene sequences in the plasma samples also tested positive in the DNA prepared from PBMCs and/or whole blood. On the other hand, only about half of the patients with MLV-related virus *gag* gene sequences detected in PBMC DNA also had viral *gag* RNA sequences detected in the plasma.

MLV-Related Viral *gag* Gene Sequences Detected in the Blood of Healthy Volunteer Blood Donors. DNA originating from 44 healthy volunteer blood donors was tested in parallel by nested PCR (Fig. 2). The nested PCR testing using the MLV-related virus *gag* gene-specific primer sets could produce many side products from human DNA (Figs. 1 and 2). We sequenced all of the PCR-amplified DNA bands from the 44 control samples of blood donors having molecular sizes close to that of the predicted PCR products from the target XMRV *gag* gene in the first round of PCR (Fig. 2A) and in the second round of nested PCR (Fig. 2B). After the first round of amplification in nested PCR, a positive PCR product with the predicted size of ~730 bp was found in PBMC DNA from 1 of 44 blood donor controls (lane 4, Fig. 2A). This ~730-bp PCR product amplified from the blood donor (BD22) was confirmed by DNA

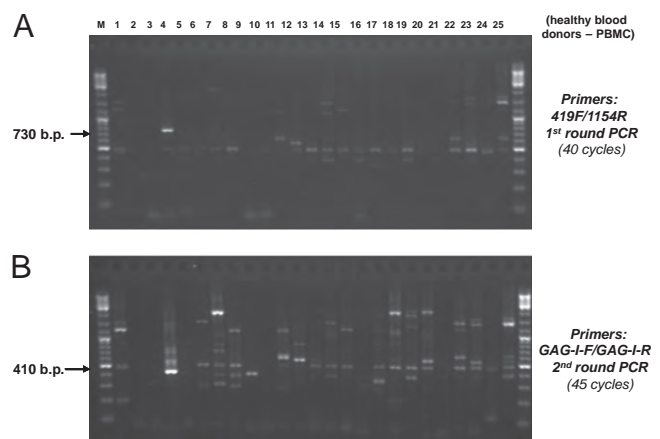


Fig. 2. MLV-related *gag* gene sequences detected in normal blood donors by nested PCR. (A) Results of PBMC DNA from blood donors 1–25 (of 44 donors examined) amplified after the first round of nested PCR using primer set 419F/1154R. Lane 4: PBMC DNA from BD22 has a positive target PCR amplicon confirmed by sequencing. (B) Results of PBMC DNA from the 25 normal blood donors after the second round of nested PCR using PCR primer set GAG-I-F/GAG-I-R (4). Sequencing of the PCR bands with size ~413 bp revealed that lane 4 (BD22), lane 7 (BD26), and lane 9 (BD28) were MLV-like virus *gag* gene sequences; lane 8 (BD27) was a human sequence. The positions of expected sizes of the positive PCR amplicons are indicated by arrows. M, DNA ladder size markers.

sequencing as an MLV-related virus *gag* gene sequence of 745 bp (Fig. S1). Overall, we found 3 of 44 (6.8%) blood donors’ PBMCs (BD22, BD26, and BD28) to be positive for the MLV-related virus *gag* gene sequences by completing both rounds of nested PCR (Fig. 2B and Fig. S2).

MLV-Related Viral *env* Gene Sequences Detected in the Blood of a CFS Patient and a Healthy Blood Donor. PBMC DNA from all of the CFS patients and healthy blood donors was also tested by PCR, targeting various regions of the MLV-related viral *env* gene. The MLV-related viral *env* gene segment of 240 bp was amplified and confirmed by sequencing from one healthy donor (BD-26) by a semi-nested PCR using the primer set 5922F/6273R in the first round of amplification and 5922F/6173R in the second round of amplification. The MLV-related viral *env* gene segment of 206 bp was amplified and confirmed by sequencing from 1 CFS patient by a nested PCR using primer set 5922F/6273R in the first round of amplification and 5942F/6159R in the second round of amplification (SI Materials and Methods).

Phylogenetic Analyses of MLV-Related Virus *gag* and *env* Gene Sequences. Multiple sequence alignment (MSA) and phylogenetic analysis of the MLV-related virus *gag* gene sequences amplified from 21 CFS patient samples and one blood donor (BD22) are shown in Fig. S1 and Fig. 3A, respectively. There were three different MLV-related retroviral *gag* gene sequences identified by PCR in the blood samples of the CFS patients and a fourth variant was detected in blood donor BD22. The sequences in all four variants were more closely related to the sequences of polytropic mouse endogenous retroviruses (mERVs) than to those of XMRVs. Although variations were observed, the majority (18/21, 86%) of CFS patient samples had the same viral *gag* gene sequence (CFS type 1), whereas 2/21 had a different, but similar, viral *gag* gene sequence (CFS type 2), and a third distinct sequence (CFS type 3) was found in the remaining CFS case. Phylogenetic analysis using the 746-nt sequences obtained revealed that CFS type 1, CFS type 2, and CFS type 3 formed a cluster that is clearly separable from the cluster formed by the newly reported XMRVs (Fig. 3A). Interestingly, the 745-nt virus *gag* gene sequence of donor BD22 (with a 1-nt deletion in the alignment) could not be included in either the cluster of CFS type 1/CFS type 2 or the cluster of

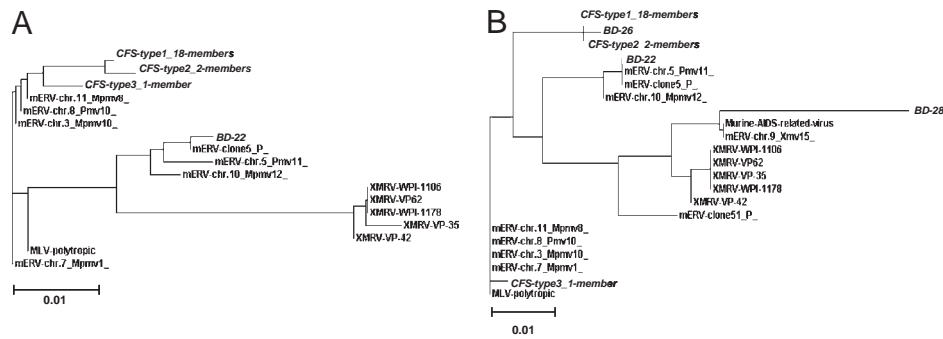


Fig. 3. Phylogenetic trees corresponding to the MSAs shown in Figs. S1 and S2 were generated by the ClustalW2 program using the neighbor-joining method (*Materials and Methods*). (A) Phylogenetic analysis based on the 746-nt *gag* gene nucleotide sequences amplified from blood samples of CFS patients and BD-22 of the corresponding MSA in Fig. S1. (B) Phylogenetic analysis based on the 380-nt *gag* gene sequences amplified from blood samples of CFS patients and healthy blood donors using the primer set NP116/NP117 of the corresponding MSA in Fig. S2.

XMRVs. The viral *gag* gene sequences of CFS type 3 and blood donor BD22 appear to be phylogenetically more closely related to polytropic mERVs or modified polytropic mERVs (Fig. 3A).

Sequence analysis of the shorter fragments of the viral *gag* gene amplified from blood of 36 out of 41 CFS patient samples and 3 out of 44 blood donor samples after the second round of nested PCR similarly confirms that there are significant variations among the MLV-like *gag* gene sequences. Fig. S2 shows sequence alignment of the 380-nt segments of viral *gag* genes amplified from three blood donors (BD22, BD26, and BD28), patients with CFS types 1–3, XMRVs, and other closely related mERVs/polytropic MLVs. As an example, the viral *gag* gene sequence identified in BD28, but not sequences of BD22 and BD26, has a prominent 21-nt deletion that is uniquely present in polytropic mERV clone 51 (Fig. S2). In phylogenetic analysis, the 380-nt segment of the *gag* gene sequence found in BD26 appears to be closely related to those of CFS types 1 and 2. However, similar to the analytic result with the 746-nt product, the 380-nt *gag* gene sequences of BD22 and BD28 again cannot be included in either the cluster of CFS type 1/CFS type 2 or the cluster of XMRVs (Fig. 3B). On the other hand, when the protein sequences coded by the *gag* gene sequences identified in the CFS patients and blood donors are compared with those of a wider range of exogenous and endogenous MLVs, they are most similar to those of polytropic MLVs and XMRVs (Fig. S3). A ClustalW Gag protein tree again reveals that MLV-like virus *gag* proteins have much more similarity to those of modified-polytropic and polytropic mERVs or to those of XMRVs, but are very different from those of exogenous ecotropic MLVs (Fig. 4).

The sequence alignment and the phylogenetic analysis of the MLV-related virus *env* gene sequences obtained from both the CFS patient and healthy blood donor revealed that they were also more closely related to those of polytropic or modified polytropic MLVs than to those of XMRVs (Fig. S4).

Testing for the Presence of Mouse DNA in Patient and Blood Donor Samples Positive for MLV-Like *gag* Gene Sequences. Mouse DNA contains endogenously many closely related proviruses of MLVs. Hence, contamination of the blood samples or reagents by mouse DNA could have produced falsely positive PCR results. Although we took great precautions to prevent potential contamination in the laboratory, and although multiple negative controls were always included in each assay, we took additional steps to confirm that no mouse DNA had contaminated the assays or the clinical samples prepared in this study. We estimated that there were about 200–1,800 mitochondrial DNA (mtDNA) copies per mammalian cell. A highly sensitive PCR assay targeting mouse-specific mtDNA was developed (*Materials and Methods*) to exclude any possible minute mouse DNA contamination in the assay system and in the clinical samples with positive amplified *gag* gene products.

The first round of the semi-nested PCR (40 cycles) used primer set mt15982F/mt16267R and could detect 10 fg of mouse DNA in

the presence of 35 ng of human background DNA. By comparison, when studied in parallel under the same assay conditions, the first round of nested PCR (40 cycles) against the MLV *gag* gene required ~10 pg of mouse DNA to detect viral *gag* gene sequences (Fig. 5A). Thus, the first round of mouse-specific mtDNA PCR assay could detect a positive mtDNA signal at a mouse DNA concentration 1,000-fold below the concentration required to detect a positive *gag* signal. The second round of mouse mtDNA semi-nested PCR, using primer set mt16115F/mt16267R, could consistently amplify the target 153-bp amplicon from 2.5 fg of mouse DNA mixed with 35 ng of human DNA (Fig. 5B). By comparison, the second round of the nested *gag* gene PCR produced positive ~400-bp amplicons from 500 fg of mouse DNA mixed with 35 ng of human background DNA in the reaction. Thus, the second round of mouse mtDNA semi-nested PCR had a sensitivity hundreds-fold higher than that of the second round of the MLV *gag* gene nested PCR in amplifying mouse DNA.

Using this highly sensitive PCR assay for mouse-specific mtDNA, we examined all of the blood samples that were found positive for MLV-like virus *gag* gene sequences from both CFS patients and healthy controls for evidence of mouse DNA contamination. PBMC DNA (30–40 ng) from the CFS patients and the healthy blood donors, as well as serial dilutions from 50 to 1 fg of mouse DNA mixed with 35 ng of human DNA as the

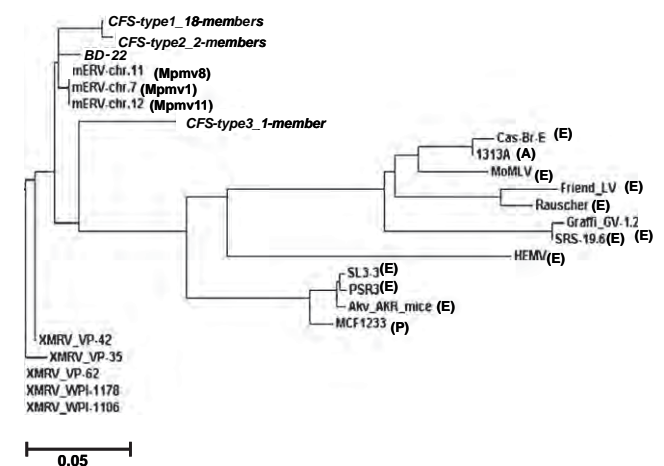


Fig. 4. Phylogenetic analysis of protein sequences based on the alignment shown in Fig. S3. CFS types 1, 2, and 3 and BD-22 and MLVs *gag* protein sequences are compared. Gag protein sequences starting from the AUG initiation codon are aligned with those of relevant endogenous as well as exogenous MLVs. Sequences of MLVs are referred to as polytropic (P), ecotropic (E), amphotropic (A), or modified polytropic (Mpmv). MoMLV, Moloney murine leukemia virus; HEMV, hortulanus endogenous murine virus.

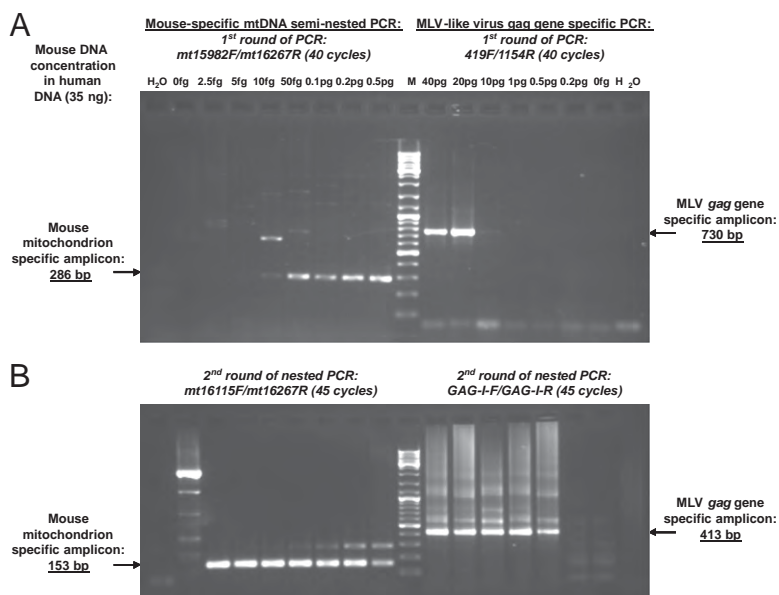


Fig. 5. Comparison of sensitivity in amplifying mouse DNA by the semi-nested PCR targeting mouse-specific mtDNA and by the nested PCR targeting MLV-like virus *gag* gene. Serial dilutions of mouse spleen DNA (from 40 pg to 2.5 fg) were spiked into 35 ng of total human PBMC DNA and compared in parallel for the mouse DNA detection sensitivity of the two PCR assays. (A) In the first round of the mtDNA-specific PCR assay, 10 fg or more of mouse DNA could be detected in the presence of 35 ng of human DNA by producing the 286-bp target product. In the first round of MLV *gag* gene nested PCR assay, 10 pg or more of mouse DNA could be detected in the presence of 35 ng of human DNA by producing the ~730-bp target product. (B) In the second round of mouse-specific mtDNA semi-nested PCR, the 153-bp target amplicon could consistently be amplified from 2.5 fg of mouse DNA. In the second round of *gag* gene-specific nested PCR, the 413-bp target product could be amplified from 0.5 pg or more of mouse DNA. Lane 0 fg: 35 ng of human DNA without spiking any mouse DNA. Lane H₂O: No DNA template. M: 100-bp DNA ladder mix. Primers and PCR cycle numbers used in each round of amplification for both of the assays are shown at the top of each gel.

positive templates, were tested in parallel. No mouse DNA was found in the PCR mix nor in the blood samples of CFS patients and blood donors that tested positive for the MLV-like virus *gag* gene sequences. Fig. 6 shows the results of the two rounds of mouse-specific mtDNA semi-nested PCR testing in DNA from PBMCs of four CFS patients (patients 8, 17, 20, and 25) with positive 746-bp amplicons in the first round of the nested PCR targeting the MLV-like virus *gag* gene, as well as from three blood donors (BD22, BD26, and BD28) who tested positive and two donors (BD21 and BD23) who tested negative for MLV-like *gag* gene sequences.

Discussion

Detection of MLV-Related Nucleic Acid Sequences. Our laboratory detected MLV-related virus *gag* gene sequences in DNA from PBMC and whole-blood samples from 32 of 37 (86.5%) CFS patients, compared with 3 of 44 (6.8%) volunteer blood donors, using a two-round nested PCR. Following only one round of PCR amplification, 21 of the 41 CFS patients' DNA samples were found positive compared with only 1 of 44 donor samples. In every instance throughout these studies, the "positive" result by PCR (an amplicon of the predicted size) was confirmed by sequencing.

In four CFS patients from whom two samples were obtained, 2 y apart, the *gag* gene sequences were detected on both occasions. Further, *gag* gene sequences were still detectable in seven of eight CFS patients from whom fresh samples were obtained ~15 y after they were initially found to be MLV *gag* gene positive. In one *gag*-positive CFS patient and one *gag*-positive blood donor, MLV-related *env* gene sequences also were detected by PCR. However, we were unable to PCR amplify and determine the MLV-related *env* gene sequences in the majority CFS patients, possibly because of the low copy number and the greater genetic variability in the *env* gene compared with the *gag* gene.

In the CFS patients, plasma samples revealed MLV-related virus *gag* gene sequences in 42% when tested by RT-PCR for viral RNA. Whereas all but one patient whose plasma tested positive for viral RNA also tested positive in PBMCs for viral DNA, only half of the cases in which MLV-related virus *gag* gene sequences were detected in PBMCs had detectable RNA sequences in plasma. Thus, accurate determination of the prevalence of these agents in patients and donors requires cellular DNA for analysis.

Sequence Variability. Previous reports of XMRV isolates from patients with CFS and with prostate cancer and from individuals in different geographic locations have described very similar nucleic acid sequences (3, 4, 12), a feature believed to be a unique

characteristic of XMRVs (13). However, our analysis revealed three different types of MLV-related virus *gag* gene sequences in CFS patients. In all three groups, the sequences were more closely related to the sequences of polytropic mERVs than to XMRVs and were more distant from the sequences of ecotropic MLVs (Fig. 3). Moreover, viral *gag* gene sequences with significant variations from both the cluster of CFS type 1/CFS type 2 and the cluster of XMRVs were identified in at least two blood donors (BD22 and BD28); phylogenetic analysis revealed the latter sequences to be more closely related to those of polytropic or xenotropic mERVs (Fig. 3B). It is unclear whether the sequence variations of the viral genes identified in the CFS patients and healthy blood donors have any significance in viral pathogenesis or disease development.

The MLV-like virus *gag* gene sequences in the CFS patients and blood donors had a deletion of 9 nt in the 5' *gag* leader region and did not have the 24-nt deletion in this region reported in XMRVs. Internal deletions of 9 nt similar to what we have identified in the CFS patients and the blood donors are known to be present in the region that encodes the glycosylated *Gag* protein (GlycoGag) in some infectious endogenous (ecotropic) MLVs and exogenous (xenotropic) MLVs, such as AKV and DG-75 (14, 15). Many previous studies have shown that the nonstructural GlycoGag of MLVs plays a critical role in viral pathogenesis and in vivo infectivity (16–19). In this context, the MLV-like virus *gag* gene sequences identified in most of our CFS patients (both CFS type 1 and CFS type 2) appear to have an intact GlycoGag in-frame with the matrix and are consistent with the gene sequences of infectious MLVs. Unfortunately, the sequences presently obtained in the study are still a bit short and lack the alternative start codon CUG. Slight extension of the 5' leader sequence will be needed to confirm the intact nature of GlycoGag.

Could Our PCR Results Have Been Falsely Positive? Voisset and coauthors (20) recently reviewed the pitfalls encountered in the identification of new retroviruses ("retrovirus"). False-positive results can occur for a variety of reasons. Viral gene sequence-specific PCR primers can nonspecifically amplify nucleic acid sequences that differ from the target sequence. For this reason, we sequenced every positive PCR product (every amplicon of the predicted size) and confirmed MLV-related gene sequences in every instance.

Although contamination must always be a concern for any PCR-based study, several pieces of evidence argue against the possibility that the PCR products identified in our study are the

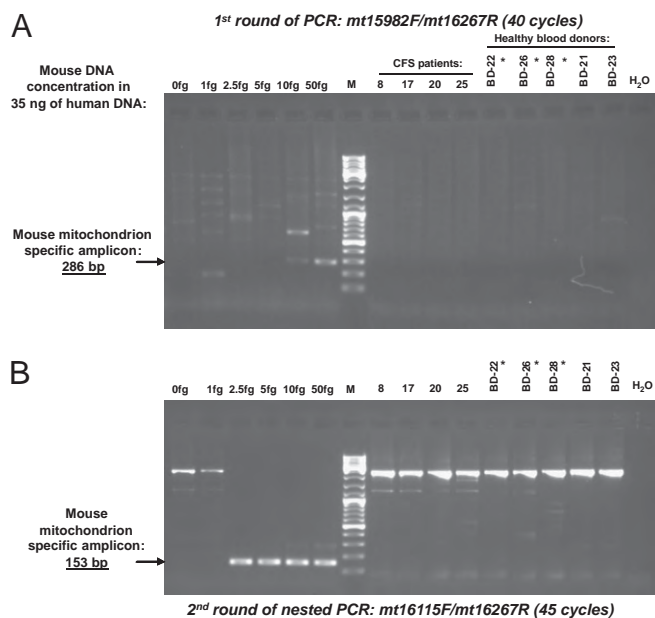


Fig. 6. Testing of CFS patients' and healthy blood donors' samples positive for MLV-like *gag* gene sequences for the presence of mouse DNA contamination using the semi-nested PCR assay targeting mouse-specific mtDNA. Serial dilutions of mouse DNA were spiked into 35 ng of human DNA and used as the controls of the assay sensitivity. The first round of mouse mtDNA semi-nested PCR (A) detected 10 fg of mouse DNA, and the second round of the semi-nested PCR (B) detected 2.5 fg of mouse DNA in the presence of 35 ng human background DNA. No evidence of mouse DNA contamination could be found by either round of mouse mtDNA semi-nested PCR in the PBMC DNA (35 ng) of CFS patients (patients 8, 17, 20, and 25); three blood donors (BD-22, BD-26, BD-28) tested positive and two blood donors (BD-21 and BD-23) tested negative for the MLV-like virus *gag* gene sequences. Healthy blood donors' samples positive for MLV-like *gag* gene sequences are labeled by asterisks. Lane 0: 35 ng of human DNA without spiking any mouse DNA. Lane H₂O: No DNA template. M: 100-bp DNA ladder mix.

result of laboratory contamination. First, every clinical sample that tested positive for the MLV-like virus *gag* gene sequences was tested for evidence of mouse DNA contamination using a semi-nested PCR for mouse-specific mtDNA that was exponentially more sensitive in detecting mouse mtDNA than MLV-related *gag* sequences (Fig. 5). Any detection of MLV *gag* that was caused by contamination with mouse DNA also would have detected mouse mtDNA by PCR, thereby identifying the *gag* result as falsely positive. In fact, no positive signal was detected by the mtDNA semi-nested PCR assay in any of the reaction mixtures or in the DNA of clinical samples examined in the study, thus excluding possible contamination by mouse DNA.

Second, we addressed the possibility that the clinical samples or the assay system might have been contaminated. The blood samples were obtained in clinical laboratories that never worked with mice or retroviral vectors and were drawn through sterile needles into vacuum tubes that remained unopened until testing. The laboratory in which PCR testing was performed also had never worked with murine cells, tissues or serum samples, or MLV vectors. Finally, because repeated entry into samples would increase the chance of contamination, we emphasize that sample vials from both patients and normal donors had never been entered before our testing.

Third, there were at least six different MLV-related *gag* gene sequences amplified from the blood samples of CFS patients and blood donors. Typically, contamination would be manifest as the same sequence in all or most samples. Moreover, the sequences that we observed all had significant variations from the previously reported exogenous MLVs or viral vectors. Furthermore, during this study more than 300 negative controls set up for

multiple PCR amplification assays targeting the MLV *gag* gene were performed, and all were negative.

Finally, a new set of blood samples was recently obtained from 8 of the original 25 patients followed in an academic medical center. Testing of the repeat blood samples, ~15 y after the index sample, showed that seven of eight patients examined had detectable MLV-*gag* gene sequences. Significant variations of MLV-like virus *gag* gene sequences amplified from the freshly obtained blood samples were not identified as would be expected in retroviral infections, but not from contamination.

The ultimate proof of low-grade infection by MLV-related viruses in humans may rely on demonstrating the integration of the viral genes into the human genome (20). The identification of provirus integration sites will take more time and effort to investigate, given that we estimate only one virus gene copy in every 400–4,000 nucleated PBMCs. Also, previous work with XMRV indicates that integration sites are quite variable (21) and the same may be true for the polytropic mouse endogenous retroviruses, which are predominant in this study.

Why Have Other Studies Come to Different Conclusions? Although we find evidence of a broader group of MLV-related viruses, rather than just XMRV, in patients with CFS and healthy blood donors, our results clearly support the central argument by Lombardi et al. (3) that MLV-related viruses are associated with CFS and are present in some blood donors. However, four recent studies have failed to confirm the PCR results reported by Lombardi (7–10). There are various possible explanations for this disparity. As stated in the reports, there could be a difference in the prevalence of these infectious agents in CFS patient populations in different geographic areas. This argument is somewhat less plausible since the publication of a recent negative study with subjects from the United States (10). Nevertheless, the heterogeneity in *gag* gene sequences that we observed suggests that geographic differences in different MLV-related viruses may be considerable and could affect both the sensitivity and the specificity of molecular amplification using standard primer sets.

Indeed, it is possible that the PCR primers used in various studies may have different sensitivity in detecting the diverse group of MLV-related virus *gag* gene sequences that we found in the clinical samples. The 5' *gag* leader sequence of previously described XMRVs represents the most divergent segment of the XMRV genome in comparison with the genomes of the other MLVs (4). In particular, there is evidently a unique 15-nt deletion in the 5' *gag* leader region in all of the XMRVs previously identified in patients with prostate cancer and CFS (3, 4). To detect XMRVs in human samples with better sensitivity and specificity, some studies used a PCR primer spanning this unique deletion as the "XMRV-specific" primer (6). However, none of the viral *gag* gene sequences amplified from the blood samples of CFS patients and blood donors in our study has this particular deletion (Fig. S1). As a consequence, such primers might have been insensitive in detecting the MLV-related *gag* gene sequences that we have identified.

Finally, it is also quite possible that there is heterogeneity in the patients diagnosed with CFS in different studies. CFS is a syndrome defined exclusively by a group of nonspecific symptoms and thus has an ill-defined phenotype. Future studies should adhere to consensus case definitions such as that developed by the Centers for Disease Control and Prevention (CDC) (1). Conversely, putative "healthy" control subjects should explicitly deny the presence of those symptoms that constitute the case definition of CFS. Furthermore, even bona fide cases of CFS may have different viral or other etiologies.

Further Considerations. The finding of XMRV or MLV sequences in persons with CFS or other diseases does not constitute definitive proof of viral infection. However, in the study of Lombardi et al. (3) and studies reviewed subsequently by Silverman et al. (22) the evidence for XMRV infection in humans not only involved detection of viral nucleic acids using PCR, but also reported the detection of

viral antigens, detection of anti-viral antibodies, the ability to culture the virus in a prostate cancer cell line, the detection of gamma retrovirus particles by electron microscopy, and transmission of infection to macaques. In sum, none of the four studies that have failed to confirm the PCR evidence reported by Lombardi et al. (3), nor our own study, has attempted to fully replicate that study.

It remains to be shown that the association that we have found, using the methods that we have described, can be generalized to a larger group of patients with CFS. Indeed, we suspect that the association will be lower in CFS cases identified through community-based surveys, as contrasted to cases seen at academic medical centers. Even if subsequent studies confirm an association between MLV-like viruses and CFS, that will not establish a causal role for these viruses in the pathogenesis of this illness. For example, such a high frequency of infections with MLV-related viruses in patients with CFS could reflect an increased susceptibility to viral infections due to an underlying CFS-related immune dysfunction, rather than a primary role for these viruses in the pathogenesis of CFS.

Finally, the finding of MLV-related virus *gag* gene sequences in nearly 7% of healthy volunteer blood donors in our study and of XMRV in 3.7% of healthy controls in the study of Lombardi et al. (3) raises additional issues. The possibility that these agents might be blood-transmitted and pathogenic in blood recipients warrants extensive research investigations of appropriately linked donor–recipient cohorts.

Materials and Methods

Samples from CFS Patients and Healthy Controls. Initially, we tested 41 whole-blood samples that had been obtained for culture isolation of mycoplasma agents in the mid-1990s. We maintained whole-blood, PBMC, and plasma samples from CFS patients in frozen storage at -80°C . Of the 41 patient samples, 29 were collected from 25 patients by one of us (A.L.K.) at the Chronic Fatigue Research Center, Brigham and Women's Hospital (Boston, MA). Four of the CFS patients also had blood obtained on a second occasion ~ 2 y later. Most of the patients were from the New England area; none were related, and virtually none had any regular social contact. Each of the 25 patients was systematically evaluated with a standardized history (supplemented by a patient questionnaire), physical examination, and battery of laboratory tests. Each met the 1988 CDC criteria for CFS, and 21 also met the 1994 CDC criteria. The average age of the patients at the time of venipuncture was 44.4 y; 4 were male and 21 were female. All of the patients signed informed consent documents approved by the Institutional Review Board of Brigham and Women's Hospital. A new set of blood samples was obtained in 2010 from 8 of the original 25 patients followed in the academic medical center. The blood samples were processed for PCR study without first being frozen. The other 12 samples from CFS patients were sent by individual clinicians taking care of patients in the mid-1990s who were given

the diagnosis of CFS. We do not have details regarding the methodology by which the referring clinicians established the diagnosis of CFS. The samples had also been sent in the mid-1990s and stored at -80°C . Frozen PBMC samples from 44 normal blood donors from the Washington, DC, area were collected in 2003–2006 and stored at the Department of Transfusion Medicine, Clinical Center, National Institutes of Health. All patient and control samples were coded and tested in parallel. Details of the preparation of blood samples and DNA/RNA isolation are described in *SI Materials and Methods*.

XMRV/MLV *gag* Nested PCR. The nested PCR for the *gag* gene was performed according to the protocols described previously (3, 4) with minor modifications. Three primer sets used in the study are as follows: 419F and 1154R (3), GAG-I-F and GAG-I-R (4), NP116 forward, and NP117 reverse. The NP116/ NP117 was an in-house–designed primer set based on the highly conserved sequences found in different MLV-like viruses and XMRVs (Fig. S1). Primer sequences and details of PCR sensitivity and specificity quality controls can be found in *SI Materials and Methods*.

Phylogenetic Analysis. To generate the neighbor-joining phylogenetic tree, the viral *gag* gene sequences obtained from blood samples of patients with CFS, normal blood donors, as well as all of the closely related MLV *gag* gene sequences selected from the National Center for Biotechnology Information (NCBI) database by BLAST querying with the *gag* gene sequences obtained in the PCR study (*SI Materials and Methods*) were aligned with ClustalW2 (<http://www.ebi.ac.uk/tools/clustalw2>) using default settings. The analysis produced the same phylogenetic trees with or without consideration of the sequence gaps in alignment.

Mouse Mitochondrial DNA Assay. The complete mtDNA sequences of humans and mice were downloaded from GenBank and aligned using ClustalW. Sequence alignment revealed the 439 bp of the 3' end of mouse mtDNA (beyond 15,862 bp, according to the coordinates of BALB/c mouse; accession no. AJ512208) were not present in human mtDNA. Primer sets were designed for a semi-nested, mouse-specific mtDNA PCR based on the sequence in this region of mouse mtDNA using Primer-Blast from NCBI. The external PCR primers (*SI Materials and Methods*) were designated as mt15982F and mt16267R, which would produce a predicted amplicon of 286 bp. The internal primers of the semi-nested PCR were designated as mt16115F and mt16267R, which would produce a predicted amplicon of 153 bp from mouse mtDNA. PCR system and setup were the same as for the *gag* gene-nested PCR study. However, PCR conditions were slightly different: 4 min at 94°C ; (30 s at 94°C , 30 s at 55°C , 1 min at 72°C) \times 40–45 cycles; 10 min at 72°C .

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Failure to Detect Xenotropic Murine Leukemia Virus–Related Virus in Blood of Individuals at High Risk of Blood-Borne Viral Infections

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(See the article by Danielson et al, on pages 1470–1477, the brief report by Henrich et al, on pages 1478–1481, and the editorial commentary by Kearney and Maldarelli, on pages 1463–1466.)

A xenotropic murine leukemia virus–related virus (XMRV) has recently been reported in association with prostate cancer and chronic fatigue syndrome, with a prevalence of up to 3.7% in the healthy population. We looked for XMRV in 230 patients with human immunodeficiency virus type 1 or hepatitis C infection. XMRV was undetectable in plasma or peripheral blood mononuclear cells by polymerase chain reaction targeting XMRV *gag* or *env*. T cell responses to XMRV *Gag* were undetectable in peripheral blood mononuclear cells by ex vivo gamma interferon enzyme-linked immunospot assay. In our cohorts, XMRV was not enriched in patients with blood-borne or sexually transmitted infections from the United Kingdom and Western Europe.

Xenotropic murine leukemia virus–related virus (XMRV) is a gammaretrovirus which has been linked with prostate cancer [1, 2] and chronic fatigue syndrome [3]. XMRV was first de-

scribed in prostate tumor samples from the United States [1, 2], but the virus could not be detected in a subsequent survey of 589 German patients with prostate cancer [4]. Two other surveys in Europe [5] and in Ireland [6] also failed to find the virus. The reason for the discrepancy is not clear. More recently, XMRV was detected in 67% of patients with chronic fatigue syndrome in the United States [3], however 4 additional studies examining cases of chronic fatigue syndrome diagnosed in Europe and the United States identified no cases of XMRV infection [7–10].

In one of these, Groom et al [8] also looked for neutralizing antibodies to XMRV and found that ~1% of serum samples tested had evidence of neutralization that could not be dismissed as nonspecific binding. These and other data suggest the prevalence of XMRV in the healthy population may lie between 1% and 3.7% [3, 8]. This has potential implications for blood transfusion recipients and is currently under investigation by the Centers for Disease Control and Prevention and the Department of Health and Human Services in the United States.

The route of transmission of XMRV has not been elucidated. We identified individuals who were at high risk for either sexually transmitted or blood-borne infections, according to whether they were infected with human immunodeficiency virus type 1 (HIV-1) or hepatitis C. We tested plasma and peripheral blood mononuclear cells (PBMCs) to reveal whether these patients were at increased risk of XMRV infection.

Methods. Two cohorts of HIV-1–infected individuals were tested for XMRV, 1 for patients with acute and 1 for those with chronic HIV-1 infection (Table 1). The cohort of patients with chronic infection included Swiss participants from the Swiss-Spanish Intermittent Therapy Trial, described elsewhere [11]. All patients were not receiving therapy at the time of sampling and had received a median duration of 26 months (range, 8.5–44.5 months) of highly active antiretroviral therapy at study entry. The median time not receiving therapy for the cohort was 14 months (range, 3–19 months). The second HIV-1–infected group of patients were recruited from a study of acute HIV-1 infection at St. Mary's Hospital, London [12]. Plasma samples were obtained from these individuals prior to receipt of antiretrovirals. For both cohorts, plasma samples and PBMCs were stored in –80°C freezers and liquid nitrogen, respectively, for up to 6 years.

The cohort of hepatitis C–positive individuals was recruited from the John Radcliffe hospital, Oxford, United Kingdom (Table 1). All patients had chronic infection and were treatment

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Table 1. Patient Cohort Characteristics

Characteristic	Patient cohort		
	Chronic HIV-1 infection ^a (n = 133)	Acute HIV-1 infection ^a (n = 101)	HCV infection (n = 67)
Sex, ratio male:female	92:41	92:9	53:14
Age, median years (range)	40 (22–68)	31 (20–67)	50 (29–81)
Available for testing	84	79	67
CD4 count pretherapy, median cells/ μ L (range)	359 (1–1035)	510 (90–1480)	...
Viral RNA pretherapy, median log ₁₀ copies/mL (range)	4.41 (2.23–6.11)	4.95 (1.90–5.95)	...
Antiretroviral therapy			
Received dual therapy	10	0	...
Received triple therapy	123	0	-
Route of acquisition or risk factor			
MSM	50 (38)	89 (88)	...
Heterosexual	60 (45)	12(12)	...
IVDU	23 (17)	0 (0)	51 (76)
Blood transfusion	10 (15)
Other ^b	9 (13)

NOTE. Data are no. or no. (%) of patients, unless otherwise indicated. HCV, hepatitis C virus; HIV-1, human immunodeficiency virus type 1; IVDU, intravenous drug user; MSM, men who have sex with men.

^a Patients with chronic HIV-1 infection were from the Swiss-Spanish Intermittent Therapy Trial cohort, and those with acute HIV-1 infection were from St Mary's Hospital acute HIV-1 cohort.

^b Other includes tattoos (6), acupuncture (1), blood product recipient (1), and vaccination (1). Three patients in the HCV cohort had >1 risk factor.

naive. Enzyme-linked immunospot (ELISPOT) assays were performed using cryopreserved PBMCs ($n = 12$) or whole blood ($n = 2$), when they were performed fresh. For the 67 polymerase chain reactions (PCR), DNA was extracted from cryopreserved PBMCs ($n = 11$), fresh PBMCs ($n = 20$), or whole blood ($n = 36$). All patients gave informed consent for enrollment in their respective studies, according to the relevant ethics committees.

DNA was extracted from 3×10^6 to 5×10^6 PBMCs or whole blood (Puregene DNA extraction kit), and 200–800 ng were used in each PCR reaction. Patient plasma samples (1 mL) underwent ultracentrifugation at 21,185 g at 4°C for 1 h and was resuspended in 140 μ L of plasma. Viral RNA was extracted (Qiagen viral RNA extraction kit) and converted to complementary DNA (cDNA) with use of random decamers and Superscript II reverse transcriptase (Invitrogen). When amplifying genomic DNA, a house-keeping gene (hGAPDH) was incorporated. For each run, 2 negative water controls and a separate positive control XMRV DNA were included (XMRV plasmid VP-62, a generous gift from Dr Robert Silverman), of which PCR reactions were able to detect at least 5 plasmid copies, determined by absorbance measurements at 260 nm.

To amplify XMRV, we targeted the *gag* and *env* genes with use of nested PCR reactions described elsewhere [1, 3]. For the *gag* PCR, we used 2 μ L of DNA or cDNA template, 5 μ L of 10 \times reaction buffer (Invitrogen), 1 μ L of 50 mmol/L MgCl₂, 1 μ L of 10 mmol/L dNTPs, 0.75 μ L of 20 μ mol/L primer GAG-O-F (5'-CGCGTCTGATTTGTTTGT-3'), 0.75 μ L of 20

μ mol/L primer GAG-O-R (5'-CCGCCTCTTCTTCATGTTC-3'), 0.2 μ L of Platinum Taq (Invitrogen), and water to make up a 50- μ L final volume. Conditions were incubation at 95°C for 4 min, followed by 35 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 45 s, and a final incubation of 72°C for 7 min. From this first-round reaction, 3 μ L were used in a nested second round with use of 5 μ L of 10 \times reaction buffer (Invitrogen), 1.5 μ L of 50 mmol/L MgCl₂, 1 μ L of 10 mmol/L dNTPs, 0.75 μ L of 20 μ mol/L primer GAG-I-F (5'-TCTCGAGATCATGGACAGA-3'), 0.75 μ L of 20 μ mol/L primer GAG-I-R (5'-AGAGGGTAAGGGCAGGGTAA-3'), 0.2 μ L of Platinum Taq (Invitrogen), and water to make up a 50- μ L reaction volume. Conditions for the second round were an incubation at 95°C for 4 min, followed by 35 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 45 s, and a final incubation of 72°C for 7 min. The resulting fragment was 413 bp in size. For the GAPDH PCR reaction, the primers used were HGAPDH-F (5'-GAAGGTGAAGGTCGGAGTC-3') and HGAPDH-R (5'-GAAGATGGTGATGGGATTTC-3'), with the same reaction conditions as the first round Gag PCR.

The *env* PCR reaction included 2 μ L of DNA or cDNA template, 5 μ L of 10 \times reaction buffer (Invitrogen), 1 μ L of 50 mmol/L MgCl₂, 1 μ L 10 mmol/L dNTPs, 0.75 μ L of 20 μ mol/L primer 5922F (5'-GCTAATGCTACCTCCCTCCTGG-3'), 0.75 μ L of 20 μ mol/L primer 6273R (5'-GGAGCCCACTGAGGAATCAAACAGG-3'), 0.2 μ L Platinum Taq (Invitrogen), and water to make up a 50- μ L reaction volume. Reaction conditions were an incubation at 95°C for 4 min, followed by 45 cycles

Table 2. Assay Results for the Identification of Xenotropic Murine Leukemia Virus-Related Virus (XMRV)

Cohort, ^a assay	Disease stage	Sample	No. of samples tested	No. of positive results
HIV-1 infection				
Gag PCR	Chronic	DNA	84	0
Env PCR	Chronic	DNA	84	0
Gag RT-PCR	Acute	RNA	79	0
Env RT-PCR	Acute	RNA	77	0
ELISPOT	Acute	PBMCs	49	0
HCV				
Gag PCR	Chronic	DNA	67	0
Env PCR	Chronic	DNA	67	0
ELISPOT	Chronic	PBMCs	14	0

NOTE. HIV-1, human immunodeficiency virus type 1; PBMC, peripheral blood mononuclear cell; PCR, polymerase chain reaction; RT-PCR, reverse transcription PCR.

^a Patients with chronic HIV-1 infection were from the Swiss-Spanish Intermittent Therapy Trial cohort, patients with acute HIV-1 infection were from St Mary's Hospital acute HIV-1 cohort, and patients with chronic HCV were from the John Radcliffe Hospital viral hepatitis clinic.

of 94°C for 30 s, 57°C for 30 s, and 72°C for 45 s, and then a final incubation of 72°C for 7 min. From this first-round reaction, 3 μ L were used in a nested second round reaction with the following conditions: 5 μ L of 10 \times reaction buffer (Invitrogen), 1.5 μ L of 50 mmol/L MgCl₂, 1 μ L of 10 mmol/L dNTPs, 0.75 μ L of 20 μ mol/L primer 5942F (5'-GGGGACGATGACAG-ACACTTCC-3'), 0.75 μ L of 20 μ mol/L primer 6200R (5'-CCCATGATGATGATGGCTTCCAGTATGC-3'), 0.2 μ L Platinum Taq (Invitrogen), and water to make up a 50- μ L reaction volume. Conditions for the second round were an initial incubation at 95°C for 4 min, followed by 35 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 45 s, and a final incubation of 72°C for 7 min. The resulting fragment was 259 bp in size.

Reactions were performed under strict conditions to avoid contamination. Master mix reagents were prepared in DNA-free areas in hoods subject to UV decontamination. DNA templates were prepared and added to the reaction in separate hoods. PCR products were run on 1% agarose gels stained with ethidium bromide in a third area, away from PCR machines and hoods.

For ex vivo T cell ELISPOT assays, 96-well plates were coated with anti-interferon- γ immunoglobulin G, to which peptide pools and between 100,000 and 200,000 PBMCs were added, in duplicate. After incubation, biotinylated interferon- γ antibody was added. Following addition of a streptavidin-labelled chromogen, the number of reactive cells was counted. Sixty-six overlapping peptides covering the XMRV Gag protein were synthesized as 18-mer peptides, overlapping by 10 amino acids and tested in pools containing 11 peptides (final concentration, 2 μ g/mL of each peptide). Assay results were considered to be positive if the mean number of spot-forming units in the test wells was greater than the mean plus 3 times the standard deviation of the negative control well spot-forming units. As-

says with high background (>10 spots/well) in the negative control wells were excluded.

Results and discussion. We tested DNA extracted from PBMCs from 84 patients who were chronically infected with HIV-1. For all patients, the PCR for hGAPDH was positive, confirming the presence of amplifiable DNA. We detected no XMRV DNA by either the *env* or *gag* PCR reactions from patient samples (Table 2), whereas in all reactions, 2 positive controls containing ~5 and ~50 copies of XMRV, respectively, amplified successfully. We tested plasma from 79 drug-naive individuals recently infected with HIV-1 by reverse transcription PCR. No patient samples were positive for XMRV *gag* or *env* RNA (Table 2). Genomic DNA extracted from the blood of 67 patients with hepatitis C virus (HCV) infection was tested for the presence of XMRV *gag* and *env* viral DNA. Positive XMRV controls and the hGAPDH house-keeping gene amplified successfully, but no samples were positive for XMRV (Table 2). In summary, of 230 patients tested, 0 (95% confidence interval, 0%–1.3%) had positive results by PCR for either XMRV *env* or *gag*.

Following the absence of positive results with PCR, we developed a novel ex vivo ELISPOT assay to identify T cell responses in PBMCs to the XMRV Gag protein. T cell ELISPOT assays are a sensitive tool for detecting immune responses to infecting pathogens and have become a routine component of clinical diagnostics, for example, to screen for latent *Mycobacterium tuberculosis* infection. The PBMCs tested in an ELISPOT assay can either be ex vivo or "cultured." We chose the ex vivo approach because it is both sensitive and specific for the detection of effector T cell responses to active infections with retroviruses and other pathogens, whereas cultured ELISPOT assays are more appropriate for detecting rare or weak non-infective memory responses, for example, those induced by

vaccines. Here, we used T cell ELISPOT assays with use of 18 amino acid overlapping peptides from XMRV Gag to look for evidence of XMRV infection in PBMCs from 49 patients with acute HIV-1 infection and 14 patients with HCV infection. We targeted the XMRV Gag gene, as in other retroviral infections, the Gag protein is highly expressed and is recognized by T cells [13]. In the original report of XMRV in patients with chronic fatigue syndrome, XMRV Gag was detectable by Western blot in lysates of patient PBMCs [3]. We found no positive responses in the 63 patients studied, giving an estimated prevalence of 0% (1-sided 95% confidence interval, 0%–4.7%), providing no evidence of enrichment for XMRV infection in this cohort. As controls, PBMCs were tested with phytohemagglutinin and FEC (influenza, Epstein-Barr virus, and human cytomegalovirus) antigens. Peptides that are 18 amino acids long will bind to both human leukocyte antigen class I and class II, and the assay is designed to detect both cytotoxic T cell (CD8) and T helper cell responses (CD4). Although widely used to detect T cells targeting retroviral peptides, this is the first time ELISPOT assays have been applied for the detection of responses to XMRV, and the result must be interpreted in the context of the lack of PCR-positive samples to act as positive controls. However, patient cells were responsive to other antigens, such as phytohemagglutinin or FEC, suggesting that the lack of responses simply reflects an absence of XMRV Gag-specific T cells in our cohort.

The identification of a previously unreported retrovirus in the human population may have profound implications. The 2 classes of known human retroviruses (HIV-1 and human T-lymphotropic virus) are pathogenic, and murine leukemia virus gene therapy vectors have caused insertional mutagenesis in children treated for X-linked immunodeficiency [14]. In our cohorts of patients with HIV-1 or HCV infection, we found no evidence of XMRV infection in blood, consistent with some of the studies on chronic fatigue syndrome [7–9]. XMRV DNA has been found within malignant prostate tissue, and XMRV can be cultured in a prostate cancer cell line, LNCaP. In vitro studies show that XMRV has an affinity for prostate cancer cells but is much less infectious for other cell types [15]. We are not aware of any studies in which the PBMCs of patients with XMRV-positive prostate cells have also been tested. It is therefore possible that in a compartmentalized infection, XMRV may not be represented in blood, and our data must be interpreted in this light. However, because all the studies of patients with chronic fatigue syndrome have used PBMCs, this is the context in which our study was set.

We hypothesized that if XMRV is present in the United Kingdom or Switzerland and is spread by either sexual or blood-borne routes, we might expect to see it enriched in the blood of patients with HIV-1 or HCV infection. We did not detect XMRV in any patients. Together, the results of PCR reactions for 230 patients and the paired ELISPOT assays are

evidence that XMRV is not enriched in patients who are at risk of blood-borne and sexually transmitted infections in the United Kingdom and Western Europe.

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