A Multicenter Blinded Analysis Indicates No Association between Chronic Fatigue Syndrome/Myalgic Encephalomyelitis and either Xenotropic Murine Leukemia Virus-Related Virus or Polytropic Murine Leukemia Virus

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ABSTRACT **The disabling disorder known as chronic fatigue syndrome or myalgic encephalomyelitis (CFS/ME) has been linked in two independent studies to infection with xenotropic murine leukemia virus-related virus (XMRV) and polytropic murine leukemia virus (pMLV). Although the associations were not confirmed in subsequent studies by other investigators, patients continue to question the consensus of the scientific community in rejecting the validity of the association. Here we report blinded analysis of peripheral blood from a rigorously characterized, geographically diverse population of 147 patients with CFS/ME and 146 healthy subjects by the investigators describing the original association. This analysis reveals no evidence of either XMRV or pMLV infection.**

IMPORTANCE **Chronic fatigue syndrome/myalgic encephalomyelitis has an estimated prevalence of 42/10,000 in the United States, with annual direct medical costs of $7 billion. Here, the original investigators who found XMRV and pMLV (polytropic murine leukemia virus) in blood of subjects with this disorder report that this association is not confirmed in a blinded analysis of samples from rigorously characterized subjects. The increasing frequency with which molecular methods are used for pathogen discovery poses new challenges to public health and support of science. It is imperative that strategies be developed to rapidly and coherently address discoveries so that they can be carried forward for translation to clinical medicine or abandoned to focus resource investment more productively. Our study provides a paradigm for pathogen dediscovery that may be helpful to others working in this field.**

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Chronic fatigue syndrome (CFS), also known as myalgic encephalomyelitis (ME), is a disabling disorder characterized by persistent unexplained fatigue in association with impaired memory or cognition, muscle or joint pain, headache, sore throat, tender lymphadenopathy, and night sweats. The prevalence in the United States is estimated at 42 cases per 10,000 population, with annual direct costs for medical care as high as $7 billion (1). Given the indirect costs in lost productivity and the social costs for patients and their families, CFS/ME is an urgent challenge for clinical medicine and public health.

Although the majority of cases are sporadic, reports of geographic and temporal clusters of CFS/ME (2-5) and the observation that many subjects report a viral prodrome and symptoms consistent with an infection have led to efforts to identify causative agents. Proposed candidates have included Epstein-Barr virus, human herpesvirus 6, enteroviruses, Borna disease virus, *Borrelia burgdorferi*, *Coxiella burnetii*, *Candida albicans*, *Mycoplasma pneumoniae*, and retroviruses (3, 4, 6-9). Initial reports of the presence in blood of CFS/ME patients of xenotropic murine leukemia virus-related virus (XMRV) (10) and of polytropic murine

leukemia virus (pMLV)-related gene sequences (11) were enthusiastically received as evidence of a tractable cause for CFS/ME.

Since the index publications, clinics have been established for the treatment of ME/CFS with antiretroviral drugs and concern has been raised with respect to safety of the blood supply. The public health significance of CFS/ME and the potential for development of assays, drugs, and vaccines to diagnose, treat and prevent disease led to independent investigations into the epidemiology of XMRV and pMLV infections in CFS/ME and non-CFS/ME populations. However, the majority of studies pursued by other investigators failed to replicate the association between XMRV or pMLV and CFS/ME (12-18). Furthermore, analysis of complete XMRV genomic sequence indicated that the virus was an artifact generated by recombination of two proviruses during tumor passaging in mice (19).

Although many studies failed to replicate the XMRV/pMLV findings, none met the criteria required to rigorously test the association between infection and disease in a multicenter study based on an appropriately powered cohort of well-characterized CFS/ME subjects and matched controls. To unequivocally address this uncertainty, our study engaged the original investigators and laboratories wherein XMRV and pMLV were reported (10, 11). Prior to initiating the study, all lead investigators at clinical and laboratory sites agreed to the criteria for selecting study subjects and to the strategy for blinding investigators and distributing and analyzing samples. Laboratories used site-specific protocols optimized for molecular or serological detection of XMRV and/or pMLV.

CFS/ME case subjects and controls were recruited using rigorous diagnostic criteria at six sites of excellence in CFS/ME clinical research across the United States. Healthy control subjects frequency matched to CFS/ME subjects by sex, age (within 5 years), race/ethnicity, season at blood sampling, and geographic residence were recruited in Boston, MA; Incline Village, NV; Miami, FL; New York, NY; Palo Alto, CA; and Salt Lake City, UT.

RESULTS

Participants completed study instruments covering symptoms, medical history, and level of function and underwent physical examination and venipuncture. All CFS/ME subjects met both the 1994 Fukuda criteria and 2003 Canadian consensus criteria (20, 21), had a viral prodrome prior to the onset of CFS (≥3 of the following clinical features: fever, headache, gastrointestinal discomfort/upset, malaise, sore throat, myalgias, arthralgias, tender lymph nodes), had reduced functional status on at least two of the three subscales of the RAND36 survey (vitality subscale, <35; social functioning subscale, <62.5; role-physical subscale, <50) (22), and scored <70% on the Karnofsky performance scale of functional impairment (23). Control subjects were excluded if they had symptoms or signs of CFS/ME or had had contact with a case subject. Potential CFS/ME and control subjects were excluded for the following confounding medical conditions: serologic evidence of infection with human immunodeficiency virus, hepatitis B virus, hepatitis C virus, *Treponema pallidum*, or *B. burgdorferi*; medical or psychiatric illness that might be associated with fatigue; or abnormal serum chemistries or thyroid function tests (Table 1). Study participants were also excluded if they were pregnant, less than 3 months postpartum, lactating, or less than 18 years or more than 70 years of age. All participants provided written informed consent in accordance with protocols approved by the Institutional Review Boards for research with human subjects of each referring institution.

TABLE 1 Normal range values for blood screening tests

Test panel parameter	Normal range
Glucose	75–100 mg/dl
Blood urea nitrogen	7–20 mg/dl
Creatinine	0.6–1.2 mg/dl
Sodium	136–146 mmol/liter
Potassium	3.5–5.0 mmol/liter
Chloride	102–109 mmol/liter
Carbon dioxide	22–30 mmol/liter
Anion gap	5–17 mEq/liter
Calcium	8.7–10.2 mg/dl
Alanine aminotransferase	7–41 U/liter
Aspartate amino transferase	12–38 U/liter
Alkaline phosphatase	33–96 U/liter
Total protein	6.7–8.6 g/dl
Albumin	3.5–5.5 g/dl
Globulin	2–3.5 g/dl
Bilirubin total	0.3–1.3 mg/dl
Bilirubin indirect	0.0–1 mg/dl
Bilirubin direct	0.0–0.4 mg/dl
Complete blood count	
White blood cell	3.5–9.1 × 10^9/liter
Red blood cell	4–5.2 × 10^{12}/liter
Hemoglobin	12–15.8 g/dl
Hematocrit	
Male	50–35%
Female	48–31%
Mean corpuscular volume	79–93.3 fl
Mean corpuscular hemoglobin	26.7–31.9 pg
Mean corpuscular hemoglobin concentration	32.3–35.9 g/dl
Platelet count	165–415 × 10^9/liter
Coefficient variation of red cell distribution width	≤14.4%
Mean platelet volume	9–13 fl
Neutrophils	40–70%
Lymphocytes	20–50%
Monocyte	4–8%
Eosinophilia	0–6%
Basophils	0–2%
Nucleated red blood cells	0%
Erythrocyte sedimentation rate	<50 mm/h
Serology	
Rapid plasma reagin	Negative
Human immunodeficiency virus	Negative

Aliquots of specimens collected from each subject were distributed in duplicate in a blinded fashion to the two teams who initially reported XMRV (Mikovits and Ruscetti) (10) or pMLV (FDA) (11) in CFS populations and to the team that first reported failure to replicate their findings (CDC) (18). To best replicate previous study designs, the FDA and Mikovits/Ruscetti/Hanson labs analyzed subject peripheral blood mononuclear cells (PBMC) and plasma; RNA from cultured cells was sent from the Ruscetti lab to the Hanson lab. The design and interpretation of experiments conducted in the Ruscetti and Hanson labs were guided by Mikovits. The CDC received only subject plasma. Positive controls were also distributed in a blinded fashion to all sites (spiked plasma controls containing 2,000 copies XMRV/ml; spiked PBMC controls containing 200 22Rv1 cells/ml).

After laboratory testing, data were returned to the Center for Infection and Immunity (CII) for unblinding and analysis. Subjects with two positive results in the same sample type were con-

TABLE 2 Characteristics of study population

Subject characteristic	CFS/ME cases ($n = 147$)	Controls ($n = 146$)
Age (years, mean ± SD)	51.9 ± 10.1	50.6 ± 10.1
Sex [no. of males (%)]	33 (22.4)	32 (21.9)
Ethnicity [no. (%)]		
Caucasian	139 (95.0)	137 (93.8)
Asian	1 (0.7)	2 (1.4)
Hispanic	7 (4.8)	7 (4.8)
African-American	0 (0)	0 (0)
Illness onset (years, mean ± SD)	35.5 ± 10.1	NA[a]
Illness duration (years, mean ± SD)	15.9 ± 8.5	NA
Vitality score (mean ± SD)[b]	8.3 ± 9.9	83.9 ± 11.6

[a] NA, not applicable.
[b] Scale is 0 to 100.

sidered positive for XMRV/pMLV. At the conclusion of the study, all investigators reviewed results and agreed to the report that follows.

The final sample set consisted of specimens from 147 case patients and 146 frequency-matched controls. Eighteen participants (10 cases, 8 controls) were excluded because of a disqualifying vitality score ($n = 2$) or onset date ($n = 1$), abnormal liver enzymes ($n = 5$) or thyroid tests ($n = 2$), insufficient total PBMC ($n = 1$), loss to follow-up ($n = 3$), or specimen thawing during transport to the coordinating laboratory ($n = 4$). Mean age and distributions of race/ethnicity, season at blood sampling, and geographic residence were comparable for case and control groups. The proportion of males was 22% among cases and controls (Table 2). Mean age at illness onset was 35.5 ±10.1 years. Cases had a mean duration of illness of 15.9 ± 8.5 years. Mean vitality score of cases on the RAND36 scale was 8.3 ± 9.9.

Testing in the CDC, FDA, and Mikovits/Ruscetti/Hanson laboratories by PCR detected the presence of XMRV and pMLV gene fragments in spiked positive-control samples. None of the plasma samples from cases were PCR positive for the presence of XMRV or pMLV at the FDA ($n = 121$) or CDC ($n = 147$). None of the plasma samples from controls were PCR positive for XMRV or pMLV at the FDA ($n = 110$) or CDC ($n = 146$). None of the uncultured PBMC from cases ($n = 121$) or controls (111) were PCR positive for XMRV or pMLV at the FDA. PCR testing by the Mikovits/Ruscetti/Hanson group of cultured PBMC from patients ($n = 117$) and controls ($n = 126$) was negative for all specimens (Table 3). The prevalence of plasma antibodies reactive with XMRV in plasma was similar in CFS/ME cases (9 of 147, or 6.1%) and controls (9 of 146, or 6.1%) (Table 3); in the exact Mantel-Haenszel test stratified by site, the *P* value was 1.0.

DISCUSSION

Our results definitively indicate that there is no relationship between CFS/ME and infection with either XMRV or pMLV. Indeed, we did not find any evidence of human infection with XMRV or pMLV in peripheral blood in our sample of 293 subjects. The absence of viral nucleic acid places an upper one-sided 95% confidence limit of 1% for the prevalence in the population sampled. This limit could be an underestimate if the observations were all false negatives. However, even if we suppose the presence of three true positives in 293 samples (1% prevalence) and a detection sensitivity as low as 0.80, the probability that all three true positives would test negative would be 0.008 and the probability that at least one sample would test positive would be 0.992. It is thus extremely unlikely that the failure to find any PCR-positive samples in this study was due to false-negative results. The serology results are more difficult to address given that the assay cannot be validated with plasma from humans with confirmed XMRV or MLV infection. We posit that positive results represent either nonspecific or cross-reactive binding and note that irrespective of explanation, a positive signal does not correlate with case status.

Sensitive molecular methods for microbial discovery and surveillance have enabled unique insights into biology and medicine. However, increased sensitivity for bona fide signal increases the risk that low-level contaminants may also be amplified. This can lead to spurious findings that pose challenges for public health and require an expensive and complex pathogen dediscovery process. Examples wherein authors of this paper have been engaged in this process include refutation of associations between enterovirus 71 and amyotrophic lateral sclerosis (24) and MMR vaccine and autism (25). In the case of CFS/ME, murine DNA contamination

TABLE 3 Equivalent levels of XMRV sequences and anti-XMRV antibodies in CFS (chronic fatigue syndrome) patients and matched controls

			CFS/ME cases ($n = 147$)		Controls ($n = 146$)	
Lab site	Analysis	Sample	Total studied	No. positive (%)	Total studied	No. positive (%)
CDC	RT-PCR	Plasma	147	0 (0.0)	146	0 (0.0)
FDA	RT-PCR	Plasma	121[a]	0 (0.0)	110[a]	0 (0.0)
	PCR	PBMC	121[a]	0 (0.0)	111[a]	0 (0.0)
Mikovits, Ruscetti, and Hanson	PCR of cultured PBMC	PBMC	117[b]	0 (0.0)	126[b]	0 (0.0)
Mikovits and Ruscetti	Serology	Plasma	147	9 (6.1)	146	9 (6.2)

[a] Numbers represent all samples available for analysis at that site.
[b] Fifty samples (30 cases; 20 controls) were unable to be assayed because at least one of two aliquots from each set of subject PBMC did not grow in tissue culture.

appears to be implicated in findings of XMRV and MLV. Indeed, mouse DNA and murine leukemia virus sequences have been found in commercial reverse transcription-PCR (RT-PCR) reagents (26, 27). It is imperative, therefore, to establish standardized strategies for rigorously testing the validity of molecular discoveries (28). We remain committed to investigating the pathogenesis of CFS/ME and to ensuring that the focus on this complex syndrome is maintained. Studies under way include the search for known and novel pathogens and biomarkers through deep sequencing and proteomics.

MATERIALS AND METHODS

Study population. A total sample of 293 subjects, 147 patients with CFS and 146 matched healthy control subjects, was recruited from six geographically diverse clinical sites. The sites included the Jen Center of Brigham and Women's Hospital in Boston, MA; Simmaron Research Institute in Incline Village, NV; Chronic Fatigue & Immune Disorders Research and Treatment Center in Miami, FL; the Levine Clinic in New York City, NY; the Infectious Disease Clinic at Stanford University in Palo Alto, CA; and the Fatigue Consultation Clinic in Salt Lake City, UT. Each of the six clinical sites enrolled between 20 and 30 subjects with matched controls.

Each study subject was assigned a unique study identification (ID) number and a series of linked sample ID numbers. Duplicate aliquots of samples from each subject were labeled with different ID codes (all linked to that subject's unique study ID) so that laboratory sites would not know whether samples came from the same subject or represented positive controls. Linkage between the study ID and each of the subject-specific sample IDs was retained by the biostatistics team until all laboratory studies were completed and all assay data were reported. All study data and samples received by the coordinating site at Columbia University were de-identified and under code (study ID or sample ID). Personal subject identifiers associated with study ID codes were kept in locked cabinets or on password-protected servers at each study site and were accessible only to key study personnel.

Screening. Potential case and control subjects were screened by each site with a brief telephone questionnaire. Subjects who appeared eligible for the study were scheduled for an evaluation visit at the respective study site.

CFS patients. Clinical site investigators recruited and screened subjects previously diagnosed with CFS for this study. At the study visit, potential cases completed multiple detailed study instruments covering symptoms, past medical history and level of function, and then underwent a physical examination and venipuncture. Based on a study algorithm that used physical examination findings and patient responses on the study instruments, potential case subjects were identified as meeting inclusion criteria for the study if they were between the ages of 18 and 70; met both the 1994 CDC Fukuda criteria for CFS and 2003 Canadian consensus criteria for ME/CFS; reported a viral-like prodrome prior to the onset of CFS, defined as three or more of the clinical features fever, headache, gastrointestinal discomfort/upset, malaise, sore throat, myalgias, arthralgias, and tender lymph nodes; had reduced functional status on at least two of the three subscales of the RAND36 survey (vitality subscale, <35; social functioning subscale, <62.5; role-physical subscale, <50) and $<70\%$ on the Karnofsky performance scale of functional impairment; were not pregnant, not <3 months postpartum, and not currently lactating; had none of the exclusionary criteria in the 1994 CDC Fukuda criteria for CFS and 2003 Canadian consensus criteria for ME/CFS case definitions; and had no diagnosis of past or current medical, neurologic, or psychiatric illnesses.

If subjects met the inclusion criteria, 80 ml of venous blood was obtained under each sample ID code and shipped to the coordinating laboratory for processing and division into aliquots. To reduce the potential for diurnal variation, blood samples were drawn between 10 a.m. and 2 p.m. (29). Assays included screens for human immunodeficiency virus infection (HIV), syphilis (rapid plasmin reagin [RPR] test), hypothyroidism (T4 and thyroid-stimulating hormone [TSH] tests), hematocrit, white blood cell count, erythrocyte sedimentation rate, electrolytes, glucose, blood urea nitrogen, creatinine, and transaminases. Potential cases were excluded if screening laboratory values were not within the ranges shown in Table 1 or were HIV or RPR positive.

Healthy controls. Healthy control subjects were recruited at the clinical sites through referral from a case participant or written public advertisement. Control subjects were frequency matched to case subjects by sex and age (within 5 years) as well as by geographic region of residence, season of case subject blood sampling (within 12 weeks), and race/ethnicity (Asian, white, black, Hispanic, or Pacific Islander) (Table 2). Subjects were excluded if they resided in the same household as, or had intimate contact with, a case subject.

Potential control subjects completed the same detailed instruments administered to potential case subjects inquiring about symptoms, medical history, and level of function and underwent a physical examination.

Potential control subjects were excluded if they reported the symptoms or signs of chronic fatigue syndrome as defined by the 1994 CDC Fukuda CFS criteria (21) and 2003 Canadian consensus CFS/ME criteria (20) or if they reported any exclusionary current or past medical, neurologic, or psychiatric illnesses that was not controlled within 5 years of the onset of CFS/ME.

As with potential case subjects, potential control subjects were identified as meeting the inclusion criteria according to a study algorithm based on recorded symptoms, medical history, functional status, and physical examination findings.

If potential control subjects met the inclusion criteria, 80 ml of venous blood was obtained and shipped to the coordinating laboratory, where they were aliquoted and screened. Control subjects who were otherwise eligible were excluded if screening laboratory values were not within the ranges shown in Table 1 or were HIV or RPR positive.

Informed consent. All participants provided informed, written consent in accordance with the protocols approved by the Institutional Review Boards (IRB) for research with human subjects that were associated with each referring institution: Columbia University (AAAI1037), Stanford University (21229), and Brigham and Women's Hospital (000688). Clinical sites not affiliated with an IRB received approval under the IRB at Columbia University. All participants provided informed, written consent to have their blood tested for HIV in accordance with the requirements of their respective state health departments.

Compensation. All potential case and control subjects were compensated for completing the study visit instruments and questionnaires, undergoing a physical examination, and providing a blood sample.

Instruments. Prior to the study visit, case and control subjects were screened according to a brief screening questionnaire for cases or healthy controls, respectively.

During the study visit, all case and control subjects completed instruments that systematically collected information regarding symptoms, medical history, level of function, quality and intensity of the fatigue, medications, sleep patterns, mood, and pain, including the RAND36 (generic form of the SF-36 instrument) (30), multidimensional fatigue inventory (MFI) (31), symptom inventory, and questionnaires covering demographics, medical history, and comorbid conditions. The physical examination included vital signs and examination of the skin, lymph nodes, head and neck, lungs, heart, abdomen, musculoskeletal system, and nervous system. Additionally, clinical investigators asked subjects to report their level of functioning according to the Karnofsky performance scale adapted for use in CFS (Nancy Klimas, personal communication).

Collection and processing of blood specimens. Fresh EDTA-treated whole-blood specimens were collected from participants on site, deidentified, and shipped overnight at 2 to 4°C to Columbia University for preparation of coded plasma and PBMC aliquots using lymphocyte separation medium. Two distinctly coded aliquots of the same sample were sent in random shipments to each laboratory.

Sample testing. Aliquots of blood specimens received at Columbia University were analyzed for blood chemistries and serologic tests for HIV and syphilis at Columbia University's Center for Advanced Laboratory Medicine or Quest Diagnostics. Thyroid hormone values were assessed by immunoassay in the CII laboratory (Merck/Millipore). Two distinctly coded aliquots of the same sample were sent in batched shipments for XMRV and pMLV testing to the participating laboratories.

Spiked controls. All cell culture media, cryopreservation reagents, and human samples used as control samples were prescreened for pMLV and XMRV sequences using previously described PCR assays (32). XMRV from 22Rv1 cell culture supernatants and cells were quantified using a previously described PCR assay that can detect a single XMRV copy (32). One-ml coded aliquots of human plasma were spiked with 22Rv1 supernatant containing ~2,000 copies of XMRV RNA. Individual vials of cryopreserved human PBMCs containing 3 million cells were spiked with ~200 22Rv1 cells (2,000 copies/specimen) (33). Spiked plasma samples and PBMC specimens were stored at −80°C until being shipped on dry ice to the testing labs in a blinded fashion with the clinical specimens.

Nucleic acid testing (NAT). The CDC received plasma specimens and performed several NAT assays as described in detail elsewhere (18, 27, 34-36). Briefly, 900 μl of plasma was ultracentrifuged at 45,000 rpm, and RNA was extracted from the pellet using a QIAamp viral RNA minikit (Qiagen). One negative-control human plasma specimen was included with every eleven test plasma specimens during the extraction process. Quantitative real-time RT-PCR assays (qRT-PCR) for generic pMLV/XMRV *pro* (protease) and *gag* detection were performed on RNA extracts. The AccessQuick RT-PCR system (Promega) with avian myeloblastosis virus (AMV) RT (Promega), and *Tfl* DNA polymerase (Promega) and an AgPath one-step RT-PCR kit (ABI/Ambion) with the ArrayScript RT and AmpliTaq Gold DNA polymerase were used for cDNA synthesis and amplification in the *pro* and *gag* qRT-PCR assays, respectively. A third PCR was done using the primers XPOLOF and XPOLOR, followed by a nested PCR with the primers XPOLIF and XPOLIR for the generic detection of MLV/XMRV 216-bp *pol* sequences (18, 27, 34-36). For this reaction, cDNA synthesis and amplification of RNA was done using AMV RT (Promega) and a RobustI RT-PCR kit (Finnzymes). Each PCR experiment included 20 water-only reactions to control for contamination. Positive bands of the correct size in each PCR assay were excised, purified, and sequenced. Detection of viral RNA and not DNA was verified by repeat qRT-PCR and nested-PCR testing of positive specimens with and without RT. To test for mouse DNA contamination, a quantitative real-time PCR assay for mouse intracisternal A particle (IAP) sequences was performed on samples that were PCR positive for XMRV/pMLV sequences (27). Specimens were considered positive if XMRV/pMLV RNA sequences were detected in at least two of three PCR assays and had undetectable IAP sequences or XMRV/pMLV DNA.

The FDA laboratory of Lo and colleagues performed nested RT-PCR and PCR assays as previously described, with some modifications (11). Briefly, 500 μl of plasma was lysed by mixing with 2 ml viral lysis buffer AVL (Qiagen) containing 5 μg carrier RNA, and nucleic acids were purified using a QIAamp viral RNA kit (Qiagen). cDNA synthesis and the first-round PCR were carried out with recombinant *Thermus thermophilus* (r*Tth*) DNA polymerase (Applied Biosystems) in triplicate using two-thirds of the total nucleic acids extracted from each specimen. For quality assurance, one negative-control plasma sample and one positive-control plasma sample (spiked with ~20 copies/ml of XMRV RNA from a 22Rv1 culture) were included with every 22 test plasma samples during the nucleic acid isolation process. During RT-PCR the following negative and positive controls were tested in triplicate: RNA diluent negative control, 2 copies of XMRV RNA per reaction, 6 copies of XMRV RNA per reaction, and 20 copies of XMRV RNA per reaction. Each assay run was considered valid if one of three XMRV-spiked positive-control plasma samples containing 20 copies/ml tested positive and at least two of three positive controls containing 6 copies of XMRV RNA per reaction tested positive. All negative controls always tested negative. Genomic DNA was extracted from PBMCs using a DNeasy blood and tissue kit (Qiagen). Nested PCR for MLV *gag* was performed in six replicates using 50 to 60 ng DNA and the 419F/1154R outer primer pair and GAG-I–F/GAG-I–R inner primer set using conditions as previously described (11). Internal positive controls consisted of normal human PBMC spiked with 22Rv1 cells (containing ~10 copies of XMRV genomes per cell). Each test run was considered valid if 2.5 copies of XMRV from 22Rv1 cells could be detected in all 3 triplicates by nested PCR. Any product of approximately the correct size (~746 bp for the outer primer pair and ~410 bp for the internal primer pairs) was excised, purified, and sequenced. PCR for detection of mouse mitochondrial DNA (mtDNA) was performed on all positive samples using a seminested PCR targeting the mouse-specific D-loop sequence as described previously (32). The mtDNA assay detected 3 fg of both mouse spleen DNA and EL4 mouse cell DNA used to spike into 50 ng of human DNA run in parallel as the positive controls. Detection of murine IAP sequences was also performed on all samples using an IAP PCR assay with 45 amplification cycles and a 72°C annealing temperature (37). The IAP PCR assay detected 100 fg of both mouse spleen DNA and EL4 mouse cell DNA used to spike 50 ng of human DNA and run in parallel as the positive controls (11).

PCR assays for the Mikovits/Ruscetti/Hanson group were performed in the Hanson laboratory at Cornell University. RNA and DNA extracts of cultured PBMC (see "Virus culture assays" below) were received on dry ice and stored at −80°C until use. Nucleic acid concentrations were measured on a NanoDrop spectrophotometer (Thermo, Fisher), and RNA was diluted in RNase-free water to 750 ng/14 μl. Fourteen microliters of each RNA sample was used for cDNA synthesis with a SuperScript VILO cDNA synthesis kit (Invitrogen). Precautions against amplicon and mouse DNA contamination, nested PCR with the Gag-O/Gag-I primers, and single-round *gagL* PCR with 419F/711R primers was performed as described by Lee et al. (38). Any bands of about the expected size were excised, purified with a PureLink quick gel extraction kit (Invitrogen), sequenced, and analyzed using BLASTn. Adequate amounts of RNA were available except for three *gag*-negative samples, for which less than 750 ng of RNA was available. IAP PCR with sensitivity for detection of 10 fg of mouse DNA was performed on any *gag*-positive samples to exclude specimen and tissue culture contamination (38, 39). DNA was not available for IAP PCR for six *gag*-positive cultures. All *gag*-positive cDNA samples were negative for mouse IAP sequences. PCR assays were considered positive if XMRV/MLV-like *gag* sequences were detected after amplification with either set of primers. In order for a specimen to be considered positive, both of the duplicate aliquots that were received were required to test positive with at least one set of primers.

Virus culture assays. Virus culture was performed on PBMC specimens by the Mikovits/Ruscetti/Hanson group at the Ruscetti laboratory. Briefly, 3 million PBMCs were stimulated with 200 U/ml interleukin 2 (IL-2) (rather than 20 U, which we observed to result in considerable cell death following thawing cells) and 2 μg phytohemagglutinin in RPMI containing 10% fetal bovine serum (FBS), 1% l-glutamine, and 1% penicillin-streptomycin in a T25 flask for 48 h. To reduce potential for contamination there was no coculture with LNCaP cells (32). Within the first 2 weeks, 10 to 15 million proliferating cells were then treated with 5 μM 5-aza-2-deoxycytidine for 4 to 5 days in order to inhibit DNA methylation, which could silence integrated retroviruses. The cells in two T25 flasks were pelleted and resuspended in Trizol (Invitrogen) for RNA isolation. The contents of two additional flasks were pelleted for DNA isolation with standard Trizol methods.

Serologic assays. At the Ruscetti lab, the Mikovits/Ruscetti/Hanson group performed a flow cytometry-based serologic assay modified slightly from what was previously reported (10). One million murine BaF3ER cells or BaF3ER cells expressing the recombinant spleen focus-forming virus (SFFV) envelope (Env) were incubated with 1:10, 1:100, and 1:1,000 dilutions of patient sera or control antisera for 20 min at room temperature. SFFV and XMRV are highly similar in the Env N terminus and would thus be recognized by cross-reactive antibodies in serologic assays. The

7C10 rat monoclonal antibody recognizes the N terminus of the XMRV/MLV Env and was used as a positive-control serum. XMRV Env blocks the binding of patient plasma to cell surface XMRV. Cells were washed, incubated with phycoerythrin (PE)-conjugated anti-human IgG (1:200) in FBS-PBS medium for 20 min at 4°C, and washed in cold PBS. Cells were then examined using a FACScan (Becton, Dickinson). Nonspecific reactivity to the BaF3ER cells was determined by incubating BaF3ER cells with patient sera diluted 1:10. Instrument settings were adjusted so that the center of the resulting negative histogram was in the center of the first log. Using the BaF3ER cells as a reference, live cells were determined using forward versus side scatter with the resulting plot set as negative and copied and pasted to the other samples in that group. This comparison was used to determine if the plasma was positive based on the level of fluorescence intensity of viable cells. Reactive human plasma does not always titrate with the 1:100 or 1:1,000 dilutions sometimes showing higher anti-human IgG binding to the BaF3ER-SFFV Env cells than the 1:10 dilution. Plasma specimens were determined to be positive if two of three dilutions reacted to the BaF3ER-SFFV Env cells at levels 3-fold that of the BaF3ER control cells. Data analysis was done using FlowJo software (Tree Star, Ashland, OR).

Statistical analysis. Individual test results reported for the two duplicate, coded sample aliquots received at each of the three laboratory sites were compared for concordance. If both aliquots of a set of duplicate samples were classified as leading to positive results in any single laboratory (based on each individual laboratory's criteria for defining positive and negative results for individual samples), the result for that sample in that laboratory was defined as positive. Discordance between test results for the duplicate aliquots examined at any one laboratory was defined as a negative result for that sample and for that laboratory. The linkage of the pair of samples within each set of duplicate sample aliquots was performed by the biostatistics team after all assays were complete and all laboratory data had been received. Serologic data were examined for group differences by an exact Mantel-Haenszel test, stratified by clinical site, with a nominal alpha level of 0.05.

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