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Journal Article

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Publication date:
2010-11-15

Permanent link:
https://doi.org/10.3929/ethz-b-000024494

Originally published in:
The Journal of Infectious Diseases 202(10), https://doi.org/10.1086/657167

BRIEF REPORT

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 described 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

Received 6 June 2010; accepted 29 July 2010; electronically published 11 October 2010.
Potential conflicts of interest: none reported.
Financial support: The Swiss National Science Foundation (SNF 33CSC0–108787 to The Swiss HIV Cohort Study), MRC (E.B. and J.F.), Oxford NIHR BRF (E.B. and J.F.), the Wellcome trust (R.E.P.), and the Swiss National Science Foundation (grant 324730_130865 to H.F.G.).
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The Journal of Infectious Diseases 2010; 202(10):1482–1485

0022-1899/2010/20210-0005$15.00
DOI: 10.1086/657167

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/μL (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 μL 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 μL of DNA or cDNA template, 5μL of 10× reaction buffer (Invitrogen), 1 μL of 50 mmol/L $MgCl_2$, 1 μL of 10 mmol/L dNTPs, 0.75 μL of 20 μmol/L primer GAG-O-F (5′-CGCGTCTGATTTGTTTTGTT-3′), 0.75 μL of 20 μmol/L primer GAG-O-R (5′-CCGCCTCTTCTTCATTGTTC-3′), 0.2 μL of Platinum Taq (Invitrogen), and water to make up a 50-μL 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 μL were used in a nested second round with use of 5 μL of 10× reaction buffer (Invitrogen), 1.5 μL of 50 mmol/L $MgCl_2$, 1 μL of 10 mmol/L dNTPs, 0.75 μL of 20μmol/L primer GAG-I-F (5′-TCTCGAGATCATGGGACAGA-3′), 0.75 μl of 20 μmol/L primer GAG-I-R (5′-AGAGGGTAAGGGCAGGGTAA-3′), 0.2 μL of Platinum Taq (Invitrogen), and water to make up a 50-μL 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 μL of DNA or cDNA template, 5 μL of 10× reaction buffer (Invitrogen), 1 μL of 50 mmol/L $MgCl_2$, 1 μL 10 mmol/L dNTPs, 0.75 μL of 20 μmol/L primer 5922F (5′-GCTAATGCTACCTCCCTCCTGG-3′), 0.75 μL of 20 μmol/L primer 6273R (5′-GGAGCCCACTGAGGAATCAAAACAGG-3′), 0.2μL Platinum Taq (Invitrogen), and water to make up a 50-μL 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 μL were used in a nested second round reaction with the following conditions: 5 μL of 10× reaction buffer (Invitrogen), 1.5 μL of 50 mmol/L $MgCl_2$, 1μL of 10 mmol/L dNTPs, 0.75 μL of 20 μmol/L primer 5942F (5′-GGGGACGATGACAGACACTTTCC-3′), 0.75 μL of 20 μmol/L primer 6200R (5′-CCCATGATGATGATGGCTTCCAGTATGC-3′), 0.2 μL Platinum Taq (Invitrogen), and water to make up a 50-μL 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 μg/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 then the mean plus 3 times the standard deviation of the negative control well spot-forming units. Assays 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 phytohemaglutinin 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 phytohemaglutinin 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.

Acknowledgments

We thank the patients and staff of the St Mary's Hospital Jefferiss Wing clinic, the patients and staff of the Swiss HIV cohort study involved in the Swiss-Spanish Intermittent Therapy Trial, and the patients and staff of the viral hepatitis clinic, John Radcliffe Hospital. Many thanks to Paul Klenerman and Kholoud Porter for helpful comments on the manuscript. Thanks to Dr R. Silverman for the donation of the control XMRV plasmid, VP-62.

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