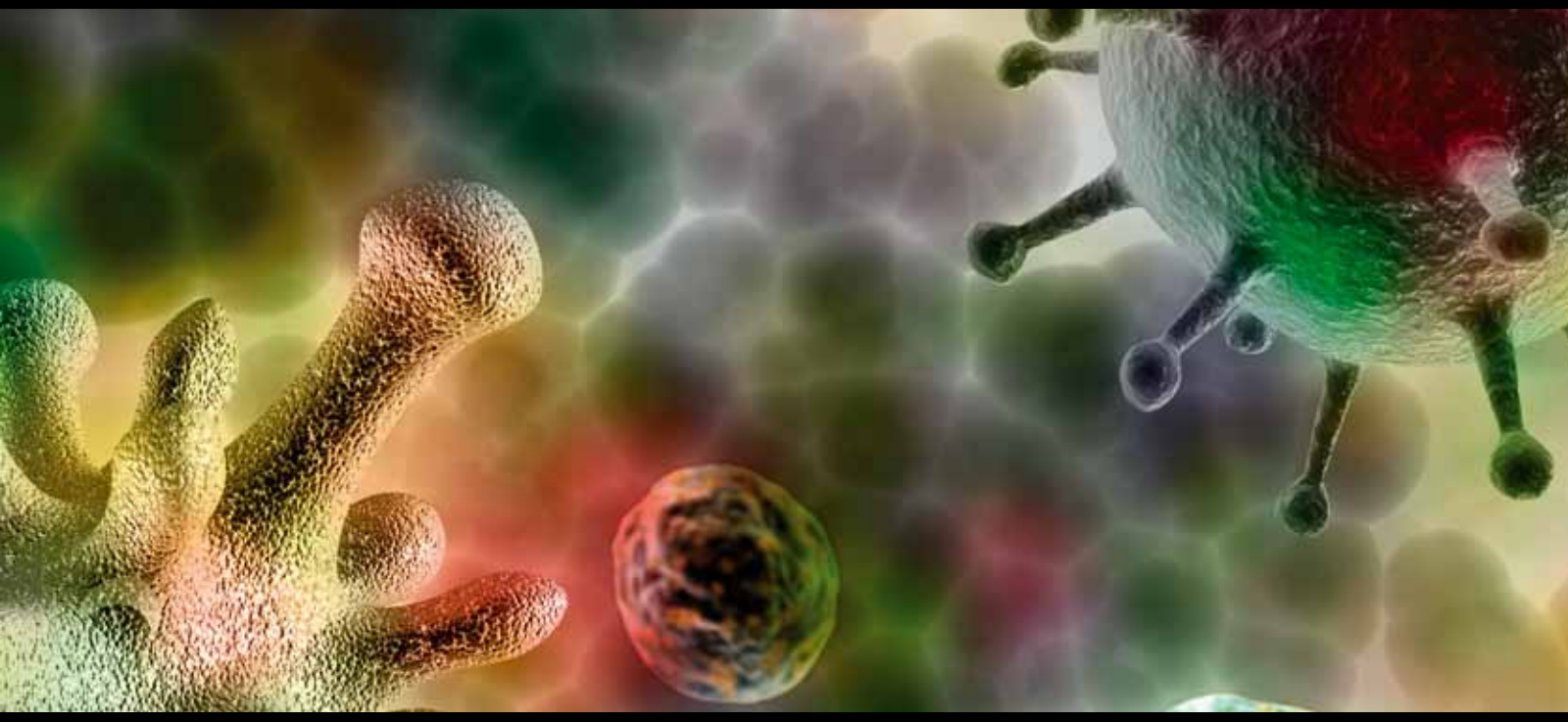
Advances in Virology

Xenotropic and Other Murine Leukemia Virus-Related Viruses in Humans

Guest Editors: Arifa S. Khan, Myra McClure, Yoshinao Kubo, and Paul Jolicoeur



Xenotropic and Other Murine Leukemia Virus-Related Viruses in Humans

Advances in Virology

Xenotropic and Other Murine Leukemia Virus-Related Viruses in Humans

Guest Editors: Arifa S. Khan, Myra McClure, Yoshinao Kubo, and Paul Jolicoeur

Editorial Board

Contents

Hindawi Publishing Corporation
Advances in Virology
Volume 2011, Article ID 787394, 2 pages
doi:10.1155/2011/787394

Editorial

Xenotropic and Other Murine Leukemia Virus-Related Viruses in Humans

Arifa S. Khan,[1] Myra McClure,[2] Yoshinao Kubo,[3] and Paul Jolicoeur[4,5,6]

[1] *Laboratory of Retroviruses, Division of Viral Products, Center for Biologics Research and Review, US Food and Drug Administration, Bethesda, MD 20892, USA*
[2] *Jefferiss Research Trust Laboratories, Wright-Fleming Institute, Faculty of Medicine, Imperial College London, Norfolk, London W2 1PG, UK*
[3] *Department of AIDS Research, Institute of Tropical Medicine, Nagasaki University, Nagasaki 852-8523, Japan*
[4] *Laboratory of Molecular Biology, Clinical Research Institute of Montreal, Qc, Canada H2W 1R7*
[5] *Department of Microbiology and Immunology, Université de Montréal, Montreal, Qc, Canada H3C 3J7*
[6] *Division of Experimental Medicine, McGill University, Montreal, Qc, Canada H3A1A3*

Correspondence should be addressed to Arifa S. Khan, arifa.khan@fda.hhs.gov

Received 5 September 2011; Accepted 5 September 2011

The recent discovery of a xenotropic murine leukemia virus-related retrovirus (designated as XMRV) in prostate cancer tissues and later in chronic fatigue syndrome (CFS) patients created excitement related to possible association of a virus with these human diseases. However, the failure to reproduce such results in other laboratories raised concerns and much debate among scientists, patient populations, and clinicians over the original findings. This special issue is a collection of recent research reports, that address this controversy. It also includes several expert reviews on various aspects of murine retroviruses, such as virus biology, replication, phylogeny, and pathogenesis, as well as XMRV in prostate cancer.

The *first* paper by A. Rein reviews the murine retrovirus genomic structure, viral structural proteins, and virus replication. The *second* paper by C. A. Kozak provides a detailed review of murine retrovirus entry into the cell and different receptor usage by different types of murine retroviruses with comparison to XMRV. The *third* paper by J. Blomberg et al. discusses the phylogenetic analysis of murine retroviruses and other retroviruses. The *fourth* paper by J. Chakraborty et al. discusses murine retrovirus pathogenesis and a mouse model for transmission of lymphoma by breast milk. The *fifth* paper by D. E. Kang et al. reviews the discovery, progress, and current status of XMRV findings in prostate cancer patients. The *sixth* paper by S. Tang and I. K. Hewlett reviews XMRV detection assays and deficiencies in the testing methods. The *seventh* paper by J. M. Coffin and O. Cingöz provides a detailed review of the controversies related to the XMRV results in human clinical samples and the findings regarding virus origin and discusses the potential sources of contamination that resulted in the misidentification of the virus as a novel human retrovirus.

Additionally, the special issue contains various research papers demonstrating the absence of XMRV in various patient populations using sensitive assays for virus detection. The *eighth* paper by B. Oakes et al. reports the absence of antibodies in CFS patients and healthy controls using two novel sensitive immunoassays. The *ninth* paper by J. Spindler et al. reports the lack of evidence of XMRV infection in HIV-1 infected men or men at high risk for HIV-1 infection by analyzing PBMCs and plasma samples using sensitive PCR assays and immunoassays. The *tenth* paper by K. A. Delviks-Frankenberry et al. demonstrates the absence of XMRV in PBMCs and plasma from HIV-1 lymphoma patients using PCR or immunoassays. The *eleventh* paper by M. J. Robinson et al. indicates the absence of XMRV sequences in prostate cancer samples from diverse populations, B-cell lymphoma patients, as well as UK blood donors. The *twelfth* paper by M. Kearney et al. reports the use of different methodologies to demonstrate the absence of XMRV in plasma and in some tissue samples from prostate cancer patients. The final, *thirteenth* paper by P. Sharma et al. describes XMRV infection

in the reproductive tissues of rhesus monkeys, proposing the possibility of an animal model for further investigations of virus transmission.

This special issue provides the recent thinking and research results of studies on XMRV and other murine leukemia retrovirus-related sequences in humans. Numerous studies have failed to confirm the presence of XMRV in humans, and XMRV has recently been found to be a laboratory-derived rare recombinant, which originated by xenografting a patient's prostate cancer cells in *nude* mice. A consensus has now emerged that XMRV footprints or infectious XMRV detected in normal human individuals or in some diseased patients represents laboratory contaminations. The information provided in this issue should be of interest to a broad audience including scientists, clinicians, patient populations, and public health agencies.

Arifa S. Khan
Myra McClure
Yoshinao Kubo
Paul Jolicoeur

Hindawi Publishing Corporation
Advances in Virology
Volume 2011, Article ID 403419, 14 pages
doi:10.1155/2011/403419

Review Article

Murine Leukemia Viruses: Objects and Organisms

Alan Rein

HIV Drug Resistance Program, National Cancer Institute-Frederick, Frederick, MD 21702, USA

Correspondence should be addressed to Alan Rein, reina@mail.nih.gov

Received 3 June 2011; Accepted 25 July 2011

Academic Editor: Arifa S. Khan

Murine leukemia viruses (MLVs) are among the simplest retroviruses. Prototypical gammaretroviruses encode only the three polyproteins that will be used in the assembly of progeny virus particles. These are the Gag polyprotein, which is the structural protein of a retrovirus particle, the Pol protein, comprising the three retroviral enzymes—protease, which catalyzes the maturation of the particle, reverse transcriptase, which copies the viral RNA into DNA upon infection of a new host cell, and integrase, which inserts the DNA into the chromosomal DNA of the host cell, and the Env polyprotein, which induces the fusion of the viral membrane with that of the new host cell, initiating infection. In general, a productive MLV infection has no obvious effect upon host cells. Although gammaretroviral structure and replication follow the same broad outlines as those of other retroviruses, we point out a number of significant differences between different retroviral genera.

1. Introduction

A virus can be viewed as a rather regular, relatively simple physical object. Alternatively, it can be seen as a living organism, evolving in response to selective pressures. Both views are correct! This paper will outline very briefly some of the characteristics of murine leukemia viruses (MLVs), keeping both views in mind. We will try to point out the distinctive features of these retroviruses, which are often taken as prototypes of the gammaretrovirus genus. (Retroviruses include Spumaretroviruses (also known as "foamy viruses") and Orthoretroviruses; the latter are divided into six genera, that is, alpha-, beta-, gamma-, delta-, epsilon-, and lentiretroviruses [1].)

MLVs have been studied for many years, beginning in the 1950s, when it was realized that leukemia could be transmitted to newborn mice by a filterable agent [2–4]. They have provided many insights into the general phenomenon of leukemogenesis. The MLV genome has also been used as the starting material in the development of vectors for gene therapy. Finally, MLVs have often been viewed as "model" retroviruses. In fact, while they have been very useful in answering questions about retroviruses and their hosts, there are many ways in which gammaretroviruses differ from other retroviruses: it should never be assumed that a given property of one genus will hold for another.

The best-studied retrovirus is, of course, human immunodeficiency virus (HIV-1), which is a lentivirus. One striking contrast between MLVs and HIV-1 is the relative simplicity of MLVs. As discussed below, MLVs only encode the proteins that will be assembled into the progeny virus particles, whereas HIV-1 encodes six additional so-called "accessory" proteins. Indeed, because of this distinction, HIV-1 has frequently been called a "complex" retrovirus, in contrast to the "simple" retroviruses such as MLV, the proper objects of study of simple retrovirologists.

The two viruses also differ in that HIV-1 can efficiently infect nondividing cells, while MLV generally does not [5, 6] (but see also [7, 8]). The ability of HIV-1 to infect nondividing cells is a critical element in its pathogenicity.

Yet another cardinal difference between MLVs and HIV-1 is that HIV-1-infected cells usually die rapidly (within a few days at most) after infection. In contrast, at the cellular level MLV infection seems almost completely benign: in general, there are no detectable effects of productive MLV infection upon the growth, physiology, or morphology of the cells. HIV-1 viremia is maintained in infected people by continual infection of new cells, replacing the cells killed by infection. We do not know how much infection is occurring in an MLV-infected, viremic mouse, but since the virus does not generally kill its host cells, the rate of new infections may be

far lower than with HIV-1. It should be noted that the drugs used in highly active antiretroviral therapy of HIV-infected people act by blocking new infections; thus, it is possible that analogous therapies would have only minimal effects on MLV viremia.

2. MLV: The Physical Object

2.1. MLV Virions. The overall structures of virus particles are probably very similar for all Orthoretroviridae. The virus is pleomorphic, but roughly spherical, with a diameter of ~100–120 nm [9]. It is released from the cell as an "immature particle", in which several thousand rod-shaped Gag polyprotein molecules are arranged, in an incomplete or imperfect hexameric lattice, as radii of the sphere (see Figure 1). The sphere is bounded by a lipid bilayer derived from the plasma membrane of the virus-producing cell. The N-terminal matrix (MA) domains of the Gag molecules are in contact with the lipid bilayer and their C-terminal nucleocapsid (NC) domains project into the interior of the particle, presumably in contact with RNA. They are approximately 20 nm long and only 2-3 nm in diameter. The particle also contains ~1–300 Gag-Pol polyprotein molecules, in which Gag is extended at its C-terminus by protease (PR), reverse transcriptase (RT), and integrase (IN). Finally, trimers of the envelope (Env) polyprotein span the membrane, with the gp70 surface glycoprotein (SU) on the exterior of the particle, complexed with the p15E transmembrane (TM) protein. Roughly 2.5×10^4 nucleotides' worth of RNA, representing only a few per cent of the mass of the particle, are also present in the virion. Some cellular proteins are also packaged: this has been documented in great detail in HIV-1 [10] but is also true in MLV [11].

After the particle is released from the cell, it undergoes maturation. PR cleaves Gag into four cleavage products, that is, MA, p12, capsid (CA), and NC. The Pol moiety of Gag-Pol is also cleaved to release free PR, RT, and IN proteins, and the C-terminal 16 residues of TM (the "R peptide") are removed, producing the mature TM protein p15E (in some papers, this shorter species is called p12E; the longer precursor has been called either p15E or Pr15E). The cleavages in Gag cause a major change in the overall architecture of the virion, with CA molecules reassembling in the interior of the particle into a polygonal structure, the "mature core" of the particle. This new structure encloses a complex of the viral RNA with NC protein; RT and IN are also presumed to be within this structure.

2.2. The MLV Replication Cycle. As with all orthoretroviruses, infection is initiated by the binding of the SU glycoprotein on the exterior of the mature, infectious virion to a receptor on the surface of the new host cell (see Figure 2). This binding event triggers dramatic changes in Env, leading to the release of the SU component and conformational rearrangement of TM. The ultimate result is the fusion of the viral membrane with the plasma membrane.

The fusion of the two membranes leads to the deposition of the contents of the virion in the cytoplasm of the cell. Once in the cytoplasm, the viral RNA is copied by the RT into a

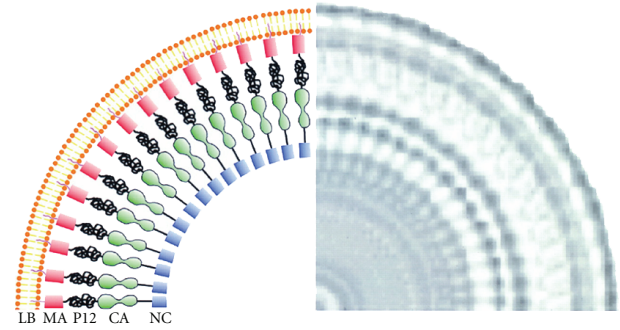


Figure 1: Structure of an immature MLV particle. A segment from a rotationally averaged cryoelectron microscopic image of a single immature MLV particle is shown on the right. As indicated on the left, the particle is bounded by a lipid bilayer ("LB"), and the MA domain of Gag (pink) is associated with the inner leaflet of the bilayer. Interior to the MA domain is a zone of low density, presumably corresponding to the p12 domain. The most conspicuous feature of the image is the "railroad tracks", representing the two domains within the CA domain (green), followed by the NC domain (blue) with bound RNA. The Pol and Env proteins are not visible in this image. As the particle is ~100 nm in diameter and the Gag molecules are ~20 nm in length, there is a region ~60 nm in diameter largely occupied by solvent in the center of the particle. (Reproduced from [9]. Copyright 1998, National Academy of Sciences, USA.)

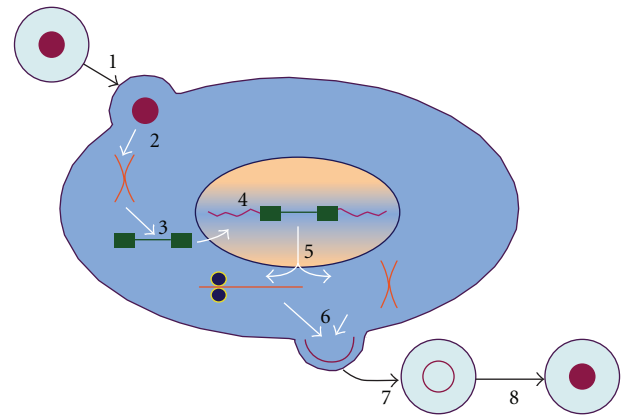


Figure 2: The orthoretroviral replication cycle. Infection is initiated when the mature, infectious virion binds to a receptor on the surface of the new host cell. The Env protein of the virus induces fusion between the viral membrane and the cell membrane (Step 1). Within the cytoplasm, the mature core dissociates (Step 2) and the dimeric viral RNA (shown in orange) is copied (Step 3) into double-stranded DNA (shown in green). The DNA copy enters the nucleus (probably when the nuclear membrane breaks down during mitosis) and is inserted into the chromosomal DNA of the cell (Step 4). The DNA is transcribed and the RNA product is exported from the nucleus (Step 5); within the cytoplasm, some molecules will be translated into viral proteins, and others are destined for encapsidation into progeny virus particles. The viral components assemble into budding virions (Step 6), which are released from the cell as immature particles (Step 7). Finally, PR cleaves the viral proteins, converting immature particles into mature, infectious virions (Step 8). It is possible that DNA synthesis actually occurs within the mature core rather than after dissociation of the core as shown here.

single molecule of dsDNA. This DNA is somehow conveyed into the nucleus, where the IN protein catalyzes its insertion into chromosomal DNA.

Once the viral DNA is integrated into host DNA, it is termed the "provirus". It is transcribed and translated by normal host-cell machinery. The encoded proteins are trafficked to the plasma membrane, where they assemble into progeny virus particles. The immature particles are released from the cell with the help of the cellular "ESCRT" machinery [23] and subsequently undergo maturation as the PR in the virus cleaves the viral polyproteins. The particle is not capable of initiating a new infection until maturation has taken place.

2.3. The MLV Genome. The RNA genome of MLV can be divided into coding and noncoding regions and is shown schematically in Figure 3.

2.3.1. Coding Regions. The only proteins encoded by the MLV genome are the three polyproteins that will make up the progeny virus particles: Gag, the structural protein of the immature virus particle, Pol, comprising the PR, RT, and IN enzymes, and Env, the SU and TM proteins that jointly mediate the entry of an infectious virus particle into a new host cell to initiate infection [18]. In some MLV isolates, an alternative form of Gag, with an N-terminal extension, is also synthesized; this "glyco-Gag" is discussed below.

As in all orthoretroviruses, the three coding regions are arranged, from 5′ to 3′, Gag : Pol : Env. The Pol proteins are initially synthesized together with Gag, in a large Gag-Pol fusion polyprotein. Gag and Gag-Pol are both translated from full-length viral RNA, identical in sequence to the genomic RNA present in the virion. It seems likely that the Gag-Pol polyprotein is incorporated into assembling virions due to "coassembly" of its Gag moiety with Gag polyprotein molecules. Successful replication of the virus requires maintenance of an optimal ratio (on the order of 20 : 1) between the Gag and Gag-Pol proteins; indeed, no detectable virus particles are formed in cells expressing only Gag-Pol [24]. This may be because Gag-Pol is more than 3 times the mass of Gag, and thus, there may not be space within the particles for very many Pol domains. This optimal ratio is achieved by finely tuned translational suppression of the termination codon at the end of the Gag coding region.

Remarkably, different retroviruses use fundamentally different mechanisms of translational suppression. In the gammaretroviruses such as MLV (and epsilonretroviruses, a genus about which very little is known), Gag and Pol are in the same reading frame, separated by a single termination codon. MLV RNA contains a 57-base cis-acting signal immediately 3′ of the termination codon [25]. This signal induces the insertion of glutamine (normally encoded by CAG), rather than termination, in response to the UAG termination codon in about 5% of the translation products; the resulting product is extended by translation of the entire Pol coding region [26]. Similar results are obtained when the UAG is replaced by UGA or UAA [27, 28]. Thus, these viruses operate in essence by "mis-translation" of the termination codon as a sense codon. In contrast, in all other genera, the suppression occurs before the ribosomes encounter the termination codon and is completely independent of this codon. In these viruses, Pol is encoded in the "−1" frame relative to Gag. A signal in these viral RNAs before the end of the Gag coding region induces a fraction of the ribosomes to advance two, rather than three bases at a specific codon so that translation by this subset of ribosomes is shifted from the Gag frame to the Pol frame [29, 30]. (In some retroviruses, there are two frameshifting events, one extending Gag to produce Gag-PR and the second extending Gag-PR to yield Gag-PR-RT-IN.) A detailed discussion of translational suppression in retroviruses may be found in Hatfield et al. [31].

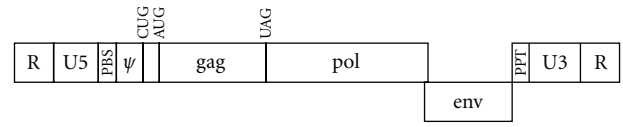


Figure 3: The MLV genome. The viral RNA in Moloney MLV is 8332 nt in length [18]. "R" sequence, 68 nt in length, is identical at both ends of the RNA. The 5′ copy of R is followed by U5 sequences and then by the PBS (nt 146–163). The long 5′ untranslated region in the RNA also includes the ψ packaging signal; contained within this signal in some MLV isolates is the CUG codon at which glyco-Gag translation is initiated (nt 357). The initiation codon for the "normal" Gag protein is at nt 621. The *gag* and *pol* coding sequences are in the same frame; they are separated by a UAG termination codon, which in turn is followed immediately by a 57-base signal, including an RNA pseudoknot, inducing the inefficient translation of the UAG as glutamine. The Env protein is translated from a spliced mRNA. The polypurine tract (PPT, nt 7803–7815) is the primer for +-strand DNA synthesis and is followed by the U3 and R regions. U3 (nt 7816–8264) is placed at the 5′ end of the DNA copy of the genome synthesized during infection; it contains promoter and enhancer sequences governing the initiation of transcription at the beginning of R.

The Env protein of MLV, like that of other orthoretroviruses, is translated from a singly spliced mRNA. There is an overlap of 58 bases between the end of the Pol coding region and the beginning of the Env coding region.

2.3.2. Noncoding Regions. Like the RNAs of all orthoretroviruses, MLV RNA also contains a set of cis-acting signals that are essential for its function as a viral genome. These include the "primer binding site" (PBS), the polypurine tract (PPT), the "packaging signal" or ψ, sequences required for insertion, by IN, of the DNA form of the viral genome into cellular DNA, and the promoter and enhancer sequences within the LTR.

The PBS is an 18-base stretch that is complementary to the last 18 bases of a cellular tRNA molecule. In MLVs, this is usually $tRNA^{Pro}$, but MLVs using $tRNA^{Gln}$ have also been found. Within the virion, the tRNA is hybridized to the viral RNA; when the virus enters a new host cell, the tRNA serves as the primer for reverse transcription. The PBS is located ~145 bases from the 5′ end of the RNA and ~460 bases 5′ of the beginning of the Gag coding region.

The first deoxynucleotide to be added to the tRNA during reverse transcription is determined by pairing with the base immediately 5′ of the PBS, and this base is the 5′ terminus of the first (minus) strand in the final DNA product. In other words, this site is the "right" end of the final double-stranded DNA product of reverse transcription.

In general, during reverse transcription the RNA is copied by the polymerase activity of RT and is progressively degraded, shortly after being copied, by the RNase H activity of RT. However, an exceptional stretch of ~15 purines near the 3′ end of retroviral RNAs (the PPT) is specifically resistant to this degradation. Having survived reverse transcription, this fragment of the viral RNA is the primer for synthesis of the second (plus) strand of DNA. The base immediately 3′ of the PPT encodes the first base of the plus strand of the DNA copy, that is, the 5′ end of the plus strand or "left" end of the double-stranded DNA.

These sequences at the two ends of the final DNA product are, of course, the sequences joined by IN to host-cell chromosomal DNA during the integration reaction. The two ends form an inverted repeat (reviewed in [32]). In Moloney MLV, the sequence of the "plus" strand at the right edge is 5′ GGGGTCTTTCA 3′, while that at the left edge is 5′ TGAAAGACCCC 3′. The bases at the 3′ ends of the plus strand on the right edge, and the 5′ end of the left edge, are joined to cellular DNA, but it is the internal bases in these sequences that are essential for IN recognition [33, 34].

All orthoretroviral genomic RNAs are, as noted above, mRNAs. They resemble cellular mRNAs in having a 5′ cap and 3′ poly (A) tail. In fact, under certain conditions, retrovirus particles can encapsidate cellular mRNAs [35]. Thus, the viral RNAs are evidently in competition with cellular mRNAs for incorporation into the virions. Intact retroviral RNAs are selectively incorporated because they contain a "packaging signal", giving them an advantage in this competition.

Recent structural studies have shed considerable light on the nature of the packaging signal in Moloney MLV RNA (see Figure 4) [20, 36]. Briefly, in all orthoretroviruses, the viral RNA is actually packaged in dimeric form, with two molecules of the viral RNA linked by a limited number of intermolecular base pairs. The primary location of these base pairs is in the "leader", between the PBS and the beginning of the Gag coding sequence. MLV RNA, like that of all gammaretroviruses, contains a pair of stem loops in this region with the sequence GACG in the loop [37]. Both NMR and chemical-probing data show that when MLV RNA dimerizes, the "CG" within each of these GACG's pairs with the CG in the other monomer (note that "CG" is a 2-base palindrome, the shortest possible palindromic sequence) [38, 39]. Further, two other stem loops in the monomers open out and pair intermolecularly. Most interestingly, this change entails a shift in register so that some of the bases which are paired in intramolecular structures in the monomers become unpaired in the dimers. These bases include two copies of the motif UCUG-UPu-UCUG. Several kinds of experiments [20] show that this motif is essential for high-affinity binding by recombinant MLV Gag protein, that these bases are occupied by NC protein within mature MLV particles, and that they are crucial to selective packaging. These results explain why dimers, but not monomers, of viral RNA are selectively packaged and also establish that the specific, high-affinity binding of Gag to ψ is responsible for selective packaging.

During reverse transcription, sequences from near the 3′ end of the viral RNA ("U3" sequences) are placed at the 5′ end, as well as near the 3′ end, of the viral DNA. (Conversely, U5 sequences, from near the 5′ end of the RNA, are placed at the 3′ end as well as near the 5′ end of the DNA.) Following integration of the viral DNA, the U3 sequences at the 5′ end constitute the promoter and enhancers driving the transcription, by Pol II, of the integrated DNA. U3 sequences include a dense collection of transcription factor-binding sites; they were used in the experiments that originally demonstrated the existence of enhancers [40] and play a major role in determining the tissue tropism and pathogenicity of the virus (reviewed in [41]). The placement of the U3 sequences, which are internal in viral RNA, upstream of the transcriptional start site in the DNA is an elegant solution to the problem of how to ensure that the viral sequences will lie 3′ of a promoter, as required for Pol II transcription.

2.4. MLV Proteins

2.4.1. Gag. In essence, the orthoretrovirus particle is constructed by assembly of Gag protein molecules. All orthoretroviral Gag proteins contain at least three domains, which will give rise to three distinct proteins in the mature virus. The MA domain at the N-terminus of Gag is responsible for targeting the protein to the plasma membrane of the virus-producing cell. In MLV, as in most retroviruses, the N-terminus of Gag is modified by the 14-carbon saturated fatty acid, myristic acid [42]; this modification is important for the plasma-membrane association of Gag [43]. The CA domain is the locus of most, if not all, of the interactions between Gag molecules leading to the assembly of the immature virion. After the CA molecules are released from the Gag polyprotein by PR, they reassemble into the mature core. The NC domain plays a predominant role in the interactions of Gag proteins with RNAs, and free NC protein is an essential cofactor in reverse transcription during infection. In general, there is considerable structural conservation between the Gag proteins in different orthoretroviral genera, despite the almost complete lack of conservation of primary sequences.

MLV Gag differs in two important respects from the canonical MA-CA-NC Gag structure (see Figure 5). First, it contains an additional domain, called p12, situated between MA and CA. p12 contains the Pro-Pro-Pro-Tyr "late domain" of MLV [44]; this motif interacts with an Nedd4-like ubiquitin ligase to promote the release of the assembled virion from the host cell [45]. p12 also participates in the infection process, but these additional functions are not well understood. It is part of the "preintegration complex", a collection of proteins from the infecting virus particle that accompany the newly synthesized viral DNA into the cell nucleus [46], and some mutations in p12 interfere with proper integration [47, 48]. Surprisingly, there are regions

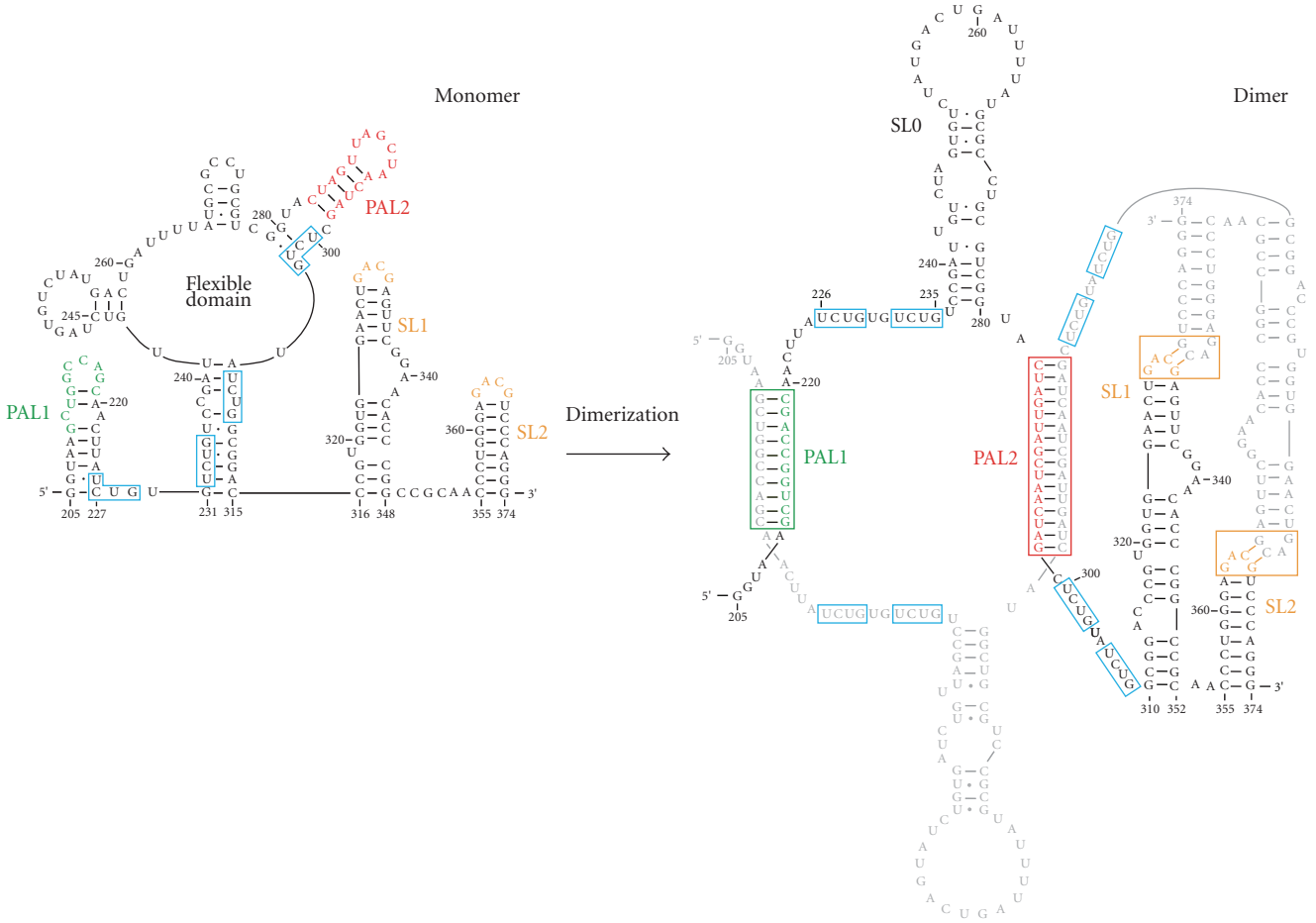


Figure 4: The Moloney MLV dimerization/packaging signal. The figure shows the secondary structure of the 170-base "minimal dimerization active sequence" (nt 205–374) [19] in both monomeric and dimeric forms. Two palindromic sequences, "PAL1" (green) and "PAL2" (red), are contained within stem loops in the monomer but open out and pair intermolecularly in the dimer. The two monomers are also connected in the dimer by base pairing between the "CG" moieties in the "GACG" loops of a pair of stem loops ("SL1" and "SL2", orange). The RNA also contains two motifs with the sequence UCUG-UPu-UCUG (blue boxes); these are partially or fully base-paired in the monomer but become unpaired as a result of the RNA rearrangements accompanying the intermolecular base pairing of PAL1 and PAL2. These bases are a crucial element in ψ, as replacement of the four UCUG sequences with UCUA prevents selective packaging of the viral RNA; the exposure of these bases in dimers, but not monomers, presumably explains the selective packaging of dimeric RNA [20]. (Figure reproduced from Trends in Biochemical Sciences, Copyright 2011, with permission from Elsevier [21].)

within p12 in which sequence changes seem to have no major effect on viral function [49, 50], and the maturation cleavage between MA and p12, unlike the other cleavages, is not absolutely essential for viral infectivity [51]. It is extremely proline-rich (18 of its 84 residues (21%) are prolines), and it has been described as "unstructured" on the basis of NMR data [52]. However, recombinant MLV Gag protein is an extended rod in solution, and the prolines in the p12 domain contribute to its rigidity (Datta et al., manuscript in preparation). It seems likely that this domain in Gag can assume any of a number of rigid conformations containing short polyproline helices.

Second, some, but not all, MLV isolates encode an alternative form of the Gag polyprotein, called "glyco-Gag" or $gPr80^{Gag}$. This protein differs in sequence from "standard" Gag in that it is extended N-terminally. Synthesis of glyco-Gag is initiated at a CUG codon in a favorable context for

Figure 5: MLV Gag protein. The MLV Gag protein is modified at its N-terminus by the 14-carbon fatty acid myristic acid. It is cleaved during virus maturation into MA, p12, CA, and NC; most of the NC molecules are also cleaved 4 residues before their C-terminus.

translation initiation, 264 bases 5′ of the normal Gag AUG initiation codon [53]. The N-terminal extension includes a signal sequence so that this protein (unlike standard Gag) is synthesized in the rough endoplasmic reticulum and processed in the Golgi apparatus. Relatively little glyco-Gag is incorporated into virions [54]. Because of a sequence polymorphism at the site of the CUG initiator, XMRV does not encode glyco-Gag.

The functional significance of glyco-Gag is still not clear. Early studies showed that it is not essential for replication of MLV in cell culture, but is needed for efficient replication and pathogenicity in mice [55, 56]. It was recently reported that the correct assembly of standard MLV Gag into spherical immature particles in cell cultures is impaired in the absence of glyco-Gag [57]; new data indicates that the presence of glyco-Gag directs virion assembly to lipid rafts and that this function involves the cellular La protein [58]. Remarkably, glyco-Gag can also complement Nef deletions in HIV-1 [59].

MLV Gag is also unusual among orthoretroviral Gags in that its NC domain only contains a single zinc finger rather than two as in most genera. The zinc-coordinating residues have the spacing C-X_2-C-X_4-H-X_4-C, as in all orthoretroviral NC proteins. This 14-residue motif plays a critical role in the selective packaging of genomic RNA, among other functions [60, 61]. The last 4 residues of NC are removed from the majority of Gag molecules, as they are from Gag-Pol molecules, during virus maturation [26, 62].

2.4.2. Pol. As noted above, the products of cleavage of the Gag-Pol polyprotein include PR, RT, and IN. PR catalyzes the cleavages leading to virus maturation; like all retroviral PRs, it is an aspartic protease which is only active as a dimer [63, 64].

RT synthesizes the DNA copy of the viral genome during infection. This function involves three enzymatic activities: RNA-templated DNA synthesis, DNA-templated DNA synthesis, and degradation of the RNA strand in an RNA:DNA hybrid, eliminating the RNA template immediately after synthesis of the complementary DNA strand. MLV RT is apparently active as a monomeric protein [65, 66] unlike the RT enzymes of alpharetroviruses and lentiretroviruses, which are both heterodimers [67].

Retroviral IN enzymes possess two catalytic activities: "3′ end processing", in which IN removes two nucleotides from the 3′ end of each strand of the DNA to be integrated, and "strand transfer", in which the new 3′ ends are inserted into chromosomal DNA in the new host cell [32]. MLV IN has not been characterized in detail but is presumed to function as a tetramer [68, 69].

2.4.3. Env. As with all orthoretroviruses, the MLV Env gene product is synthesized in the rough endoplasmic reticulum and glycosylated in the Golgi apparatus. It is also cleaved in the Golgi by a cellular furin-like protease into two fragments, the large, N-terminal surface glycoprotein ($gp70^{SU}$) and the C-terminal transmembrane protein $p15E^{TM}$. A trimer of these heterodimeric SU-TM complexes is then trafficked to the cell surface. As mentioned above, it undergoes an additional cleavage during virus maturation: PR removes the C-terminal 16 residues, also known as the "R peptide", from the cytoplasmic tail of the TM protein [62, 70]. This maturation cleavage of TM is found in the gammaretroviruses, in Mason-Pfizer monkey virus, a betaretrovirus [71, 72], and in the lentivirus equine infectious anemia virus [73], but not, as far as is known, in other retroviruses.

MLV Env is depicted schematically in Figure 6. Mature SU of Moloney MLV is 435 residues in length, while TM is 180 residues. In turn, SU contains an N-terminal "receptor-binding domain" (RBD) of ~240 residues, a short, proline-rich "hinge" region, and a highly conserved C-terminal domain [74]. The RBD consists of an antiparallel β-sandwich projecting "up" from the surface of the virion, and a highly variable region resting atop this scaffold. Both ends of the RBD contribute to this β-sandwich [75]. Sequence alignments and analysis of chimeric SU proteins show that the variable sequences within the RBD make specific contacts with cell-surface receptors. Among the conserved features of SU are a histidine residue near the extreme N-terminus and a CXXC motif in the C-terminal portion of SU. TM protein begins with a very hydrophobic stretch, the "fusion peptide". A stretch between TM residues 43 and 78 (in Moloney MLV) has a 4-3 repeating pattern of hydrophobic residues that forms a coiled coil. TM also contains a CX_6CC motif; in the virus particle, there is a disulfide bond joining SU, *via* one of the cysteines in the CXXC, to TM, *via* the last cysteine in the CX_6CC [76–78].

The function of the Env complex is to induce fusion between the membrane surrounding the virus particle and the membrane of a new host cell. As in all orthoretroviruses, the cleavage between SU and TM is absolutely required for Env function [79]. Presumably, this is essential because it places the fusion peptide at the N-terminus of TM rather than in the interior of the Env polyprotein. The removal of the R peptide from the C-terminus of Prp15E during virus maturation is also necessary for the fusogenicity of Env [80, 81]. It seems likely that fusogenic activity would be harmful to the virus-producing cell and that the R peptide is a "safety catch" suppressing this activity until the virus has left the cell. The mechanism by which the R peptide inhibits fusion is not known, but, remarkably, it has the same effect when joined to the influenza HA protein [82].

The fusion between the two membranes by the mature Env complex is the end result of an amazing cascade of events. Briefly, binding to the receptor on the plasma membrane induces a conformational change in the RBD. This change is propagated in SU, resulting in the ionization of the one free thiol in its CXXC motif [83]. (The conserved histidine near the N-terminus of SU, which is essential for Env function, may catalyze this ionization [84].) The ionized sulfur then attacks the neighboring cysteine, and the disulfide linkage between SU and TM is replaced by an intra-SU bond between these two cysteines. Breaking the SU-TM bond releases SU from the Env complex, exposing the fusion peptide at the N-terminus of TM. The fusion peptide inserts into the target membrane; this is followed by a major conformational change in TM, in which a C-terminal heptad repeat-like sequence in the TM ectodomain folds against the N-terminal heptad repeat [76]. This shift to a hairpin configuration brings the two membranes into very close apposition; this finally results in the fusion of the two membranes.

Further studies make it clear that RBD functions not only to bind a receptor on the target cell, but also to prevent the conformational change in TM, leading to membrane

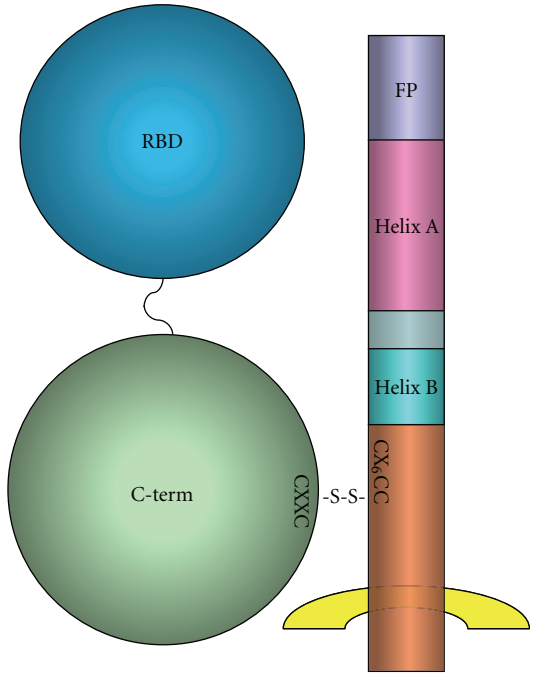


Figure 6: MLV Env protein. MLV Env protein consists of a complex between $gp70^{SU}$ and $p15E^{TM}$. The cartoon shows that gp70 has two domains, RBD at its N-terminus and "C-term" at its C-terminus, separated by a variable, proline-rich linker. P15E contains, from N- to C-terminus, the fusion peptide (FP), an N-terminal helical domain (helix A), a short C-terminal helical domain (helix B), and a C-terminal domain (light pink) which spans the viral membrane (yellow). Gp70 is exclusively external to the virus and is connected to p15E by a disulfide linkage between one of the two cysteines in a CXXC motif within its C-terminal domain and the last cysteine in a CX_6CC motif in p15E.

fusion, from occurring prematurely, that is, before contact of the virus with the receptor [85, 86]. In fact, under special circumstances infection can occur "in *trans*", that is, when a *soluble* RBD binds a cell-surface receptor in proximity to the virion [87]. This activity of the MLV Env complex has special consequences for the "MCF" class of MLVs. These "mink cell focus-inducing" or "polytropic" MLVs arise in mice that are viremic for ecotropic MLVs, and are recombinants in which the ecotropic RBD has been replaced by an RBD from an endogenous MLV genome [88–90]. This substitution gives the MCF a different receptor specificity from that of its ecotropic parent [13–15, 91]. The complex of the ecotropic SU protein with the ecotropic receptor on target cells (as in the viremic mice) has been shown to facilitate infection of the cells by MCF virions [92].

Remarkably, TM protein performs yet another function for MLV. Immediately proximal to the CX_6CC motif discussed above is a 20-residue stretch which has potent immunosuppressive activity; this activity is crucial in MLV infections in mice [93, 94].

As indicated above, MLVs are polymorphic with respect to their use of cell-surface receptors. In general, when a cell is productively infected with an MLV, the viral Env protein saturates the receptors that it would use for infection, rendering the cell almost completely resistant to superinfection by virus particles that use the same receptor. This resistance makes it possible to group MLV isolates into families sharing common receptors. "Interference" measurements of this kind showed that NIH/3T3 mouse cells have four distinct cell-surface molecules used as receptors by different MLVs, as indicated in Table 1 [91, 95]. This polymorphism is considered in detail in a comprehensive review [96], and is discussed in other articles in this series. It is notable that all receptors used by MLVs contain multiple membrane-spanning domains, unlike the known receptors for most other orthoretroviruses.

3. MLV: The Organism

3.1. Assays for Infectious MLV. Quantitative virology is virtually impossible without a reliable infectivity assay [97]. Since MLVs generally have no obvious effect on the cells they infect, the opportunities for developing a "plaque" or "focus" assay have been very limited. Two such assays have been devised, each exploiting a specific cell line with a unique response to MLV infection.

One of these is the "UV-XC" test [98]. XC cells, derived from a rat tumor induced by Rous sarcoma virus, undergo rapid syncytium formation when they come into contact with cells producing ecotropic MLV. This property was used to develop an "indirect" plaque assay: a plate of permissive cells is first infected with the virus, and the virus is allowed to spread in these cells for 5–7 days. At the end of this period, the cells have grown into a confluent monolayer, and the plate contains invisible "foci" of MLV-producing cells. Each focus has arisen by the localized spread of virus from a single cell, infected by a virus in the inoculum, to neighboring cells; several rounds of replication can occur during the assay. This monolayer is then killed by UV-irradiation and overlaid with XC cells. A day later, the XC cells have replaced the original cells; they are fixed and stained, and "plaques", that is, localized regions of syncytia, are counted. One particular advantage of this assay is that it can be used to measure the infectivity of any ecotropic MLV on any cells; thus, for example, comparing the titer of a single virus preparation on NIH/3T3 cells and Balb/3T3 cells tells one whether the virus is N-tropic, B-tropic, or NB-tropic. On the other hand, the fact that it only detects ecotropic MLVs is a serious limitation of the UV-XC test.

The other quantitative assay for replication-competent MLV is the S+L− assay [99]. S+L− cells are specific cell lines transformed by Moloney sarcoma virus. When these cells are superinfected by an MLV, they become much rounder and more refractile (this may reflect "hypertransformation", perhaps due to reinfection of the cells with additional copies of Moloney sarcoma virus after it has been rescued by the MLV). In this assay, S+L− cells are infected and allowed to grow for ∼5 days; "foci" of rounded cells, which stand out against the confluent monolayer of uninfected S+L− cells, are then scored under a low-power microscope. This assay has the advantage that it will detect any replication-competent MLV, not just members of a specific class.

Table 1: MLV receptors on NIH/3T3 mouse cells.

Virus class	Example	Receptor	Reference
Ecotropic	Moloney MLV	mCAT1	[12]
Polytropic	MCF247	XPR1	[13–15]
Amphotropic	1504A	SLC20A2	[16, 17]
10A1	10A1	SLC20A1 or SLC20A2	[16, 17]

The table lists the receptors for MLVs found on NIH/3T3 mouse cells. The diversity of MLV receptors is discussed in more detail in other articles of this series.

However, it is extremely time consuming. It can also be difficult to distinguish the foci from random irregularities in the cell monolayer, so scoring the assay requires considerable skill and involves some judgment.

For many, but not all, kinds of experiments, replication-defective "reporter" viruses rescued by MLV can be assayed in lieu of assaying the MLV itself. The reporter viruses originally used in this way were acute transforming viruses; for example, MLVs were grouped into interference families by measuring the ability of Harvey MSV pseudotypes to transform MLV-infected cells [91, 95]. More recently, of course, MLV-derived vectors expressing a variety of genes, such as luciferase, β-galactosidase, and green fluorescent protein, have been constructed for use as reporter viruses (e.g., [100]).

Cell lines have also been developed in which a reporter gene is only expressed following replication in the cell of an MLV. These cells contain an MLV-derived vector which carries a reporter gene in reverse orientation; the reporter gene is interrupted by an intron in the forward orientation. Transcription and splicing yields an RNA in the cell with an uninterrupted, negative-sense copy of the reporter gene; if this RNA is rescued by an MLV, it can be copied into DNA, finally producing an intact reporter gene whose expression can be measured (Aloia et al., manuscript in preparation, but see [101]). This assay has the special advantage that it can be performed by cocultivation of the assay cells with cells producing the virus to be assayed, as well as by infection of the assay cells with cell-free virus.

3.2. Endogenous MLVs. At least 100 times over the course of evolution, MLVs have infected cells of the mouse germline. Once the viral DNA has integrated into the germline DNA, it is passed from parents to offspring just like any other mouse gene. The biology of these "endogenous" MLVs and their effects on their hosts are quite complex and are considered in other articles in this series.

3.3. Resistance to MLV. While MLVs are generally benign at the cellular level, they do induce both lymphomas and neurological diseases in mice. Mice have evolved a number of resistance mechanisms that inhibit the growth of MLVs; MLVs have, in turn, developed strategies for evading these defense mechanisms.

3.3.1. Superinfection Interference. Two genes inducing strong resistance to specific envelope classes of MLV have been described: Fv-4 and Rmcf [102, 103]. Both of these genes have been found to function by superinfection interference: in other words, the genes encode glycoproteins which bind MLV receptors, rendering the receptors unavailable for incoming viruses. Fv-4 blocks the ecotropic receptor, mCAT1, whereas Rmcf blocks the MCF receptor XPR1. It seems reasonable to imagine that these genes were originally introduced into the mouse genome as the Env genes of endogenous MLVs.

3.3.2. Fv1 Restriction. Fv1 restriction was the first system for resistance to MLV to be described in mice [104]. Inbred mouse strains carry the "n" allele, the "b" allele, or the "nr" allele at the Fv1 locus. In turn, naturally occurring MLVs may be N-tropic or B-tropic. $Fv1^n$ or $Fv1^{nr}$ mice are partially resistant to B-tropic MLVs, while the $Fv1^b$ locus encodes partial resistance to N-tropic MLVs ($Fv1^{nr}$ mice are resistant to some N-tropic MLVs as well as B-tropic MLVs). Passage of an MLV in the restrictive host may ultimately lead to the selection of a viral variant that has lost its sensitivity to Fv1 restriction; these laboratory isolates, such as Moloney MLV, are termed NB-tropic. XMRV is unique in that it is restricted by both $Fv1^n$ and $Fv1^b$ [105].

Despite many years of investigation, the mechanism of Fv1 restriction is still not well understood. The Fv1 gene product seems to be a somewhat degenerate retroviral Gag protein [106]. Genetic data indicate that it binds to a specific site in the N-terminal domain of CA in the mature core of the incoming virus particle. This interaction blocks infection at a point between reverse transcription and integration of the viral DNA. The Fv1 protein is present in cells at extremely low levels [107]; in fact, restriction can be blocked or "abrogated" by infection with a single particle of the restricted type [108]. Particles which have been inactivated by heat or gamma irradiation can retain the ability to abrogate Fv1 restriction [109].

Biochemical analysis of the Fv1 restriction machinery has proven extremely difficult, but it appears that the ability of the Fv1 protein to multimerize [110] is an essential element in restriction [111]. The specific binding of the protein to CA protein of the restricted type seems to occur only when the mature CA is in a lattice, as in the viral core; this binding was recently demonstrated, for the first time, using CA protein arrayed on lipid nanotubes [112].

While the Fv1 restriction system is, as far as is known, found only in mouse cells, human cells possess a somewhat analogous restriction system effected by the TRIM5α protein. TRIM5α was discovered by virtue of its ability to restrict HIV-1, but it is also active against some MLVs; remarkably, like the Fv1 gene product, it distinguishes between N-tropic and B-tropic MLVs [113].

3.3.3. APOBEC3 Restriction. All placental mammals have at least one member of the APOBEC3 gene family; humans and chimpanzees have seven APOBEC3 genes [114, 115]. APOBEC3 proteins can be incorporated into retrovirus particles, and they interfere with viral replication during reverse

transcription when the APOBEC3-bearing virus particle infects a new host cell. APOBEC3s are cytidine deaminases with one or two zinc-coordinating motifs that are instrumental in the restriction of viral replication. It seems likely that the primary function of APOBEC3s is protection of the mammalian host against pathogens (or intracellular parasites such as retrotransposons): mice lacking mouse APOBEC3 (mA3) survive and reproduce normally but are very sensitive to retrovirus infection [116, 117].

One way in which APOBEC3 proteins inactivate retroviruses is by hypermutation. By deaminating deoxycytidine to deoxyuridine in minus-strand DNA during the synthesis of viral DNA, they bring about a G to A change in the plus-strand. Many susceptible viruses have been shown to incur very high levels of G to A mutation as a result of APOBEC3 action. However, it is now clear that APOBEC3 proteins act on retroviruses in other ways as well. For example, the degree of inactivation of HIV-1 by human APOBEC3G (hA3G) does not necessarily correlate with the level of G to A mutation (reviewed in [118]), and hA3G has been shown to affect both the synthesis and integration of HIV-1 viral DNA [119].

There are two isoforms of mA3, containing or lacking exon 5. Most studies on mA3 have used the form lacking the exon. MLVs show dramatic differences in their sensitivity to this mA3: both XMRV and AKV (the endogenous ecotropic MLV in AKR mice, a mouse line bred for high leukemia incidence) are far more sensitive to inactivation by mA3 than Moloney MLV (which was selected for rapid growth and leukemogenicity by passage in mice over a period of years) [105, 120–122]. Moreover, when DNA of XMRV or AKV is synthesized in the presence of mA3, it contains large numbers of G to A mutations [120, 121], but these mutations are not detectably induced in Moloney MLV by mA3 [100, 123]. Presumably, the creation of the AKR mouse strain entailed the selection of mice that provide a maximally permissive environment for AKV, and thus, this virus has not faced selective pressure leading to mA3 resistance. In contrast, selection during passage of Moloney MLV has led to partial resistance to inactivation by mA3, and apparently complete resistance to the hypermutational effects of mA3. The mechanisms underlying these resistance phenomena are unknown. It should be noted that in HIV-1, one of the "accessory proteins", that is, Vif, is responsible for viral resistance to hA3G. Vif functions by binding to hA3G and inducing its proteasomal degradation. However, as emphasized above, MLVs do not encode accessory proteins, and the resistance of Moloney MLV to mA3 must reside in its Gag, Pol, and/or Env protein. As mA3 is packaged efficiently in Moloney MLV particles [100, 123], the resistance does not depend upon exclusion of mA3 from the virus.

The biology of MLV restriction by the mA3 containing exon 5 is somewhat different from the foregoing: mA3 protein containing this exon can be cleaved by MLV PR, leading to the inactivation of this mA3 within MLV particles [124].

3.3.4. Restriction by Tetherin. Recently, yet another antiviral restriction system has been discovered, mediated by the host protein "tetherin" (also known as CD317, BST2, or HM1.24) [125]. Tetherin is a membrane protein with a very unusual topology: it has a cytoplasmic N-terminus, followed by a transmembrane helix, an extended ectodomain, and a C-terminus associated with the plasma membrane by a glycophosphatidyl inositol linkage. Tetherin dimerizes via the ectodomain, which forms a coiled coil ~90 Å long. The presence of membrane anchors at both ends of the molecule evidently gives it the ability to physically link released virus particles to the surface of the virus-producing cell, effectively preventing their escape into the surrounding medium (see Figure 7) [22].

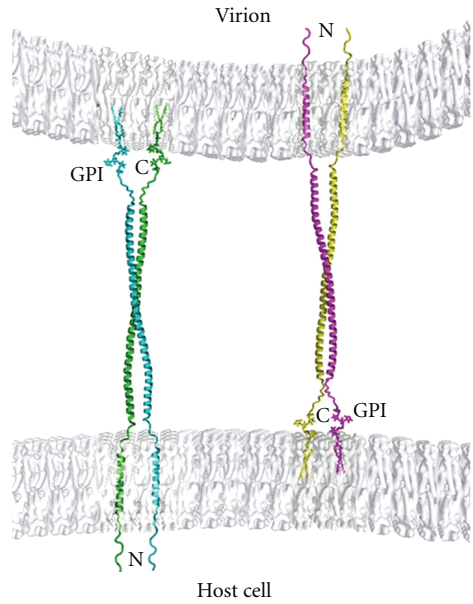


Figure 7: Hypothetical mechanism of restriction by tetherin. The cellular restriction factor tetherin can act as a bridge between two membranes. Tetherin contains a transmembrane domain at its N-terminus and is anchored to a membrane by a glycophosphatidyl linkage at its C-terminus. It also dimerizes due to a parallel coiled-coil structure between the termini of the protein. Anchorage to membranes at both ends apparently enables tetherin to "trap" virus particles, preventing their escape from the virus-producing cell. It is not known which end of the protein is embedded in the cellular membrane and which in the viral membrane. (Figure reproduced with permission from [22].)

Tetherins inhibit the release of all retroviruses tested, and also of filoviruses such as Ebola, arenaviruses such as Lassa, and herpesviruses such as Kaposi's sarcoma-associated herpesvirus. They are constitutively expressed on some cell surfaces and are inducible by type I interferon in others. Mouse tetherin has been shown to inhibit the replication of MLV [126]. While lentiviruses have several alternative countermeasures against tetherins, including the HIV-1 accessory protein Vpu (reviewed in [127]), no resistance mechanisms in MLVs have yet been described.

4. Concluding Remarks

It is clear that MLVs have provided an extraordinary wealth of information about retroviruses, both as physical objects

and as living organisms. They (and other gammaretroviruses, such as gibbon ape leukemia virus) are now being developed as vectors for gene therapy. As has been indicated throughout this paper, the contrasts with other retroviruses such as HIV-1 help to illustrate the range of possibilities by which viruses solve common problems. Finally, as with all viruses, MLVs provide a window into the "black box", an unparalleled opportunity to learn about the cells and organisms that they infect. Indeed, many cellular proteins have been shown to participate in MLV replication; while this large topic is beyond the scope of this paper, it is the focus of a fascinating review by Goff [128].

Acknowledgments

The author thanks his colleagues John Coffin and Steve Hughes for many helpful discussions and particularly Jim Cunningham for insight into MLV Env gymnastics. He also thanks Bob Bassin for his mentorship and generosity during his introduction to the study of MLVs. Research in his laboratory is supported by the Intramural Research Program of the NIH, National Cancer Institute, and Center for Cancer Research.

References

[1] M. L. Linial et al., "Retroviridae. Virus taxonomy: classification and nomenclature of viruses," in *Proceedings of the International Committee on Taxonomy of Viruses*, C. M. Fauquet, M. A. Mayo, J. Maniloff, U. Desselberger, and L. A. Ball, Eds., pp. 421–440, Elsevier/Academic Press, San Diego, Calif, USA, 2004.

[2] L. Gross, ""Spontaneous" leukemia developing in C3H mice following inoculation in infancy, with AK-leukemic extracts, or AK-embrvos," *Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine*, vol. 76, no. 1, pp. 27–32, 1951.

[3] L. Gross, "Development and serial cellfree passage of a highly potent strain of mouse leukemia virus," *Proceedings of the Society for Experimental Biology and Medicine*, vol. 94, pp. 767–771, 1957.

[4] J. B. Moloney, "Biological studies on a lymphoid-leukemia virus extracted from sarcoma 37. I. Origin and introductory investigations," *Journal of the National Cancer Institute*, vol. 24, pp. 933–951, 1960.

[5] P. F. Lewis and M. Emerman, "Passage through mitosis is required for oncoretroviruses but not for the human immunodeficiency virus," *Journal of Virology*, vol. 68, no. 1, pp. 510–516, 1994.

[6] T. Roe, T. C. Reynolds, G. Yu, and P. O. Brown, "Integration of murine leukemia virus DNA depends on mitosis," *EMBO Journal*, vol. 12, no. 5, pp. 2099–2108, 1993.

[7] L. Jarrosson-Wuilleme, C. Goujon, J. Bernaud, D. Rigal, J. L. Darlix, and A. Cimarelli, "Transduction of nondividing human macrophages with gammaretrovirus-derived vectors," *Journal of Virology*, vol. 80, no. 3, pp. 1152–1159, 2006.

[8] X. H. Liu, W. Xu, J. Russ, L. E. Eiden, and M. V. Eiden, "The host range of gammaretroviruses and gammaretroviral vectors includes post-mitotic neural cells," *PLoS ONE*, vol. 6, no. 3, Article ID e18072, 2011.

[9] M. Yeager, E. M. Wilson-Kubalek, S. G. Weiner, P. O. Brown, and A. Rein, "Supramolecular organization of immature and mature murine leukemia virus revealed by electron cryo-microscopy: implications for retroviral assembly mechanisms," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 13, pp. 7299–7304, 1998.

[10] D. E. Ott, "Cellular proteins detected in HIV-1," *Reviews in Medical Virology*, vol. 18, no. 3, pp. 159–175, 2008.

[11] J. E. Bubbers and F. Lilly, "Selective incorporation of H 2 antigenic determinants into Friend virus particles," *Nature*, vol. 266, no. 5601, pp. 458–459, 1977.

[12] L. M. Albritton, L. Tseng, D. Scadden, and J. M. Cunningham, "A putative murine ecotropic retrovirus receptor gene encodes a multiple membrane-spanning protein and confers susceptibility to virus infection," *Cell*, vol. 57, no. 4, pp. 659–666, 1989.

[13] J. L. Battini, J. E. J. Rasko, and A. D. Miller, "A human cell-surface receptor for xenotropic and polytropic murine leukemia viruses: possible role in G protein-coupled signal transduction," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 4, pp. 1385–1390, 1999.

[14] C. S. Tailor, A. Nouri, C. G. Lee, C. Kozak, and D. Kabat, "Cloning and characterization of a cell surface receptor for xenotropic and polytropic marine leukemia viruses," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 3, pp. 927–932, 1999.

[15] Y. L. Yang, L. Guo, S. Xu et al., "Receptors for polytropic and xenotropic mouse leukaemia viruses encoded by a single gene at Rmc1," *Nature Genetics*, vol. 21, no. 2, pp. 216–219, 1999.

[16] D. G. Miller and A. D. Miller, "A family of retroviruses that utilize related phosphate transporters for cell entry," *Journal of Virology*, vol. 68, no. 12, pp. 8270–8276, 1994.

[17] C. A. Wilson, K. B. Farrell, and M. V. Eiden, "Properties of a unique form of the murine amphotropic leukemia virus receptor expressed on hamster cells," *Journal of Virology*, vol. 68, no. 12, pp. 7697–7703, 1994.

[18] T. M. Shinnick, R. A. Lerner, and J. G. Sutcliffe, "Nucleotide sequence of Moloney murine leukaemia virus," *Nature*, vol. 293, no. 5833, pp. 543–548, 1981.

[19] C. S. Badorrek and K. M. Weeks, "RNA flexibility in the dimerization domain of a gamma retrovirus," *Nature Chemical Biology*, vol. 1, no. 2, pp. 104–111, 2005.

[20] C. Gherghe, T. Lombo, C. W. Leonard et al., "Definition of a high-affinity Gag recognition structure mediating packaging of a retroviral RNA genome," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 45, pp. 19248–19253, 2010.

[21] A. Rein, S. A. Datta, C. P. Jones, and K. Musier-Forsyth, "Diverse interactions of retroviral Gag proteins with RNAs," *Trends in Biochemical Sciences*, vol. 36, no. 7, pp. 373–380, 2011.

[22] H. Yang, J. Wang, X. Jia et al., "Structural insight into the mechanisms of enveloped virus tethering by tetherin," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 43, pp. 18428–18432, 2010.

[23] O. Pornillos, J. E. Garrus, and W. I. Sundquist, "Mechanisms of enveloped RNA virus budding," *Trends in Cell Biology*, vol. 12, no. 12, pp. 569–579, 2002.

[24] K. M. Felsenstein and S. P. Goff, "Expression of the gag-pol fusion protein of Moloney murine leukemia virus without gag protein does not induce virion formation or proteolytic processing," *Journal of Virology*, vol. 62, no. 6, pp. 2179–2182, 1988.

[25] Y. X. Feng, H. Yuan, A. Rein, and J. G. Levin, "Bipartite signal for read-through suppression in murine leukemia virus mRNA: an eight-nucleotide purine-rich sequence immediately downstream of the gag termination codon followed by an RNA pseudoknot," *Journal of Virology*, vol. 66, no. 8, pp. 5127–5132, 1992.

[26] Y. Yoshinaka, I. Katoh, T. D. Copeland, and S. Oroszlan, "Murine leukemia virus protease is encoded by the gag-pol gene and is synthesized through suppression of an amber termination codon," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 82, no. 6, pp. 1618–1622, 1985.

[27] Y. X. Feng, T. D. Copeland, S. Oroszlan, A. Rein, and J. G. Levin, "Identification of amino acids inserted during suppression of UAA and UGA termination codons at the gag-pol junction of Moloney murine leukemia virus," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 87, no. 22, pp. 8860–8863, 1990.

[28] Y. X. Feng, J. G. Levin, D. L. Hatfield, T. S. Schaefer, R. J. Gorelick, and A. Rein, "Suppression of UAA and UGA termination codons in mutant murine leukemia viruses," *Journal of Virology*, vol. 63, no. 6, pp. 2870–2873, 1989.

[29] T. Jacks, M. D. Power, F. R. Masiarz, P. A. Luciw, P. J. Barr, and H. E. Varmus, "Characterization of ribosomal frameshifting in HIV-1 gag-pol expression," *Nature*, vol. 331, no. 6153, pp. 280–283, 1988.

[30] T. Jacks and H. E. Varmus, "Expression of the Rous sarcoma virus pol gene by ribosomal frameshifting," *Science*, vol. 230, no. 4731, pp. 1237–1242, 1985.

[31] D. L. Hatfield, J. G. Levin, A. Rein, and S. Oroszlan, "Translational suppression in retroviral GENE expression," *Advances in Virus Research*, vol. 41, pp. 193–239, 1992.

[32] P. O. Brown, "Integration," in *Retroviruses*, J. M. Coffin, S. H. Hughes, and H. E. Varmus, Eds., pp. 161–203, Cold Spring Harbor Laboratory Press, Plainview, NY, USA, 1997.

[33] J. Colicelli and S. P. Goff, "Mutants and pseudorevertants of Moloney murine leukemia virus with alterations at the integration site," *Cell*, vol. 42, no. 2, pp. 573–580, 1985.

[34] J. Colicelli and S. P. Goff, "Sequence and spacing requirements of a retrovirus integration site," *Journal of Molecular Biology*, vol. 199, no. 1, pp. 47–59, 1988.

[35] S. J. Rulli Jr., C. S. Hibbert, J. Mirro, T. Pederson, S. Biswal, and A. Rein, "Selective and nonselective packaging of cellular RNAs in retrovirus particles," *Journal of Virology*, vol. 81, no. 12, pp. 6623–6631, 2007.

[36] V. D'Souza and M. F. Summers, "Structural basis for packaging the dimeric genome of Moloney murine leukaemia virus," *Nature*, vol. 431, no. 7008, pp. 586–590, 2004.

[37] D. A. M. Konings, M. A. Nash, J. V. Maizel, and R. B. Arlinghaus, "Novel GACG-hairpin pair motif in the 5' untranslated region of type C retroviruses related to murine leukemia virus," *Journal of Virology*, vol. 66, no. 2, pp. 632–640, 1992.

[38] C. Gherghe, C. W. Leonard, R. J. Gorelick, and K. M. Weeks, "Secondary structure of the mature ex virio Moloney murine leukemia virus genomic RNA dimerization domain," *Journal of Virology*, vol. 84, no. 2, pp. 898–906, 2010.

[39] C. H. Kim and I Tinoco Jr., "A retroviral RNA kissing complex containing only two G·C base pairs," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 17, pp. 9396–9401, 2000.

[40] B. Levinson, G. Khoury, G. Vande Woude, and P. Gruss, "Activation of SV40 genome by 72-base pair tandem repeats of Moloney sarcoma virus," *Nature*, vol. 295, no. 5850, pp. 568–572, 1982.

[41] A. B. Rabson and B. J. Graves, "Synthesis and processing of viral RNA," in *Retroviruses*, J. M. Coffin, S. H. Hughes, and H. E. Varmus, Eds., pp. 205–262, Cold Spring Harbor Laboratory Press, New York, NY, USA, 1997.

[42] L. E. Henderson, H. C. Krutzsch, and S. Oroszlan, "Myristyl amino-terminal acylation of murine retrovirus proteins: an unusual post-translational protein modification," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 80, no. 2, pp. 339–343, 1983.

[43] A. Rein, M. R. McClure, N. R. Rice, R. B. Luftig, and A. M. Schultz, "Myristylation site in Pr65(gag) is essential for virus particle formation by Moloney murine leukemia virus," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 83, no. 19, pp. 7246–7250, 1986.

[44] B. Yuan, S. Campbell, E. Bacharach, A. Rein, and S. P. Goff, "Infectivity of Moloney murine leukemia virus defective in late assembly events is restored by late assembly domains of other retroviruses," *Journal of Virology*, vol. 74, no. 16, pp. 7250–7260, 2000.

[45] C. Segura-Morales, C. Pescia, C. Chatellard-Causse, R. Sadoul, E. Bertrand, and E. Basyuk, "Tsg101 and Alix interact with murine leukemia virus Gag and cooperate with Nedd4 ubiquitin ligases during budding," *Journal of Biological Chemistry*, vol. 280, no. 29, pp. 27004–27012, 2005.

[46] A. Prizan-Ravid, E. Elis, N. Laham-Karam, S. Selig, M. Ehrlich, and E. Bacharach, "The Gag cleavage product, p12, is a functional constituent of the murine leukemia virus pre-integration complex," *PLoS Pathogens*, vol. 6, no. 11, Article ID e1001183, 2010.

[47] B. Yuan, A. Fassati, A. Yueh, and S. P. Goff, "Characterization of Moloney murine leukemia virus p12 mutants blocked during early events of infection," *Journal of Virology*, vol. 76, no. 21, pp. 10801–10810, 2002.

[48] A. Yueh and S. P. Goff, "Phosphorylated serine residues and an arginine-rich domain of the Moloney murine leukemia virus p12 protein are required for early events of viral infection," *Journal of Virology*, vol. 77, no. 3, pp. 1820–1829, 2003.

[49] M. R. Auerbach, C. Shu, A. Kaplan, and I. R. Singh, "Functional characterization of a portion of the Moloney murine leukemia virus gag gene by genetic footprinting," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 20, pp. 11678–11683, 2003.

[50] B. Yuan, X. Li, and S. P. Goff, "Mutations altering the Moloney murine leukemia virus p12 Gag protein affect virion production and early events of the virus life cycle," *EMBO Journal*, vol. 18, no. 17, pp. 4700–4710, 1999.

[51] M. Oshima, D. Muriaux, J. Mirro et al., "Effects of blocking individual maturation cleavages in murine leukemia virus Gag," *Journal of Virology*, vol. 78, no. 3, pp. 1411–1420, 2004.

[52] S. K. Kyere, P. R. B. Joseph, and M. F. Summers, "The p12 domain is unstructured in a murine leukemia virus p12-CA(N) Gag construct," *PLoS ONE*, vol. 3, no. 4, Article ID e1902, 2008.

[53] A. C. Prats, G. De Billy, P. Wang, and J. L. Darlix, "CUG initiation codon used for the synthesis of a cell surface antigen coded by the murine leukemia virus," *Journal of Molecular Biology*, vol. 205, no. 2, pp. 363–372, 1989.

[54] R. Fujisawa, F. J. McAtee, C. Favara, S. F. Hayes, and J. L. Portis, "N-terminal cleavage fragment of glycosylated Gag is incorporated into murine oncornavirus particles," *Journal of Virology*, vol. 75, no. 22, pp. 11239–11243, 2001.

[55] A. Corbin, A. C. Prats, J. L. Darlix, and M. Sitbon, "A non-structural gag-encoded glycoprotein precursor is necessary for efficient spreading and pathogenesis of murine leukemia viruses," *Journal of Virology*, vol. 68, no. 6, pp. 3857–3867, 1994.

[56] P. Schwartzberg, J. Colicelli, and S. P. Goff, "Deletion mutants of Moloney murine leukemia virus which lack glycosylated gag protein are replication competent," *Journal of Virology*, vol. 46, no. 2, pp. 538–546, 1983.

[57] A. Low, S. Datta, Y. Kuznetsov et al., "Mutation in the glycosylated gag protein of murine leukemia virus results in reduced in vivo infectivity and a novel defect in viral budding or release," *Journal of Virology*, vol. 81, no. 8, pp. 3685–3692, 2007.

[58] T. Nitta, R. Tam, J. W. Kim, and H. Fan, "The cellular protein La functions in enhancement of virus release through lipid rafts facilitated by murine leukemia virus glycosylated Gag," *mBio*, vol. 2, no. 1, 2011.

[59] M. Pizzato, "MLV glycosylated-Gag is an infectivity factor that rescues Nef-deficient HIV-1," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 20, pp. 9364–9369, 2010.

[60] R. J. Gorelick, W. Fu, T. D. Gagliardi et al., "Characterization of the block in replication of nucleocapsid protein zinc finger mutants from Moloney murine leukemia virus," *Journal of Virology*, vol. 73, no. 10, pp. 8185–8195, 1999.

[61] R. J. Gorelick, L. E. Henderson, J. P. Hanser, and A. Rein, "Point mutants of Moloney murine leukemia virus that fail to package viral RNA: evidence for specific RNA recognition by a "zinc finger-like" protein sequence," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 85, no. 22, pp. 8420–8424, 1988.

[62] L. E. Henderson, R. Sowder, T. D. Copeland, G. Smythers, and S. Oroszlan, "Quantitative separation of murine leukemia virus proteins by reversed-phase high-pressure liquid chromatography reveals newly described gag and env cleavage products," *Journal of Virology*, vol. 52, no. 2, pp. 492–500, 1984.

[63] L. Menendez-Arias, D. Gotte, and S. Oroszlan, "Moloney murine leukemia virus protease: bacterial expression and characterization of the purified enzyme," *Virology*, vol. 196, no. 2, pp. 557–563, 1993.

[64] R. Swanstrom and J. W. Wills, "Synthesis, assembly, and processing of viral proteins," in *Retroviruses*, J. M. Coffin, S. H. Hughes, and H. E. Varmus, Eds., pp. 263–334, Cold Spring Harbor Laboratory Press, Plainview, NY, USA, 1997.

[65] D. Das and M. M. Georgiadis, "The crystal structure of the monomeric reverse transcriptase from Moloney murine leukemia virus," *Structure*, vol. 12, no. 5, pp. 819–829, 2004.

[66] K. Moelling, "Characterization of reverse transcriptase and RNase H from friend-murine leukemia virus," *Virology*, vol. 62, no. 1, pp. 46–59, 1974.

[67] A. Telesnitsky and S. P. Goff, "Reverse transcriptase and the generation of retroviral DNA," in *Retroviruses*, J. M. Coffin, S. H. Hughes, and H. E. Varmus, Eds., pp. 121–160, Cold Spring Harbor Laboratory Press, Plainview, NY, USA, 1997.

[68] S. Hare, F. Di Nunzio, A. Labeja, J. Wang, A. Engelman, and P. Cherepanov, "Structural basis for functional tetramerization of lentiviral integrase," *PLoS Pathogens*, vol. 5, no. 7, Article ID e1000515, 2009.

[69] S. Hare, S. S. Gupta, E. Valkov, A. Engelman, and P. Cherepanov, "Retroviral intasome assembly and inhibition of DNA strand transfer," *Nature*, vol. 464, no. 7286, pp. 232–236, 2010.

[70] A. Schultz and A. Rein, "Maturation of murine leukemia virus env proteins in the absence of other viral proteins," *Virology*, vol. 145, no. 2, pp. 335–339, 1985.

[71] B. A. Brody, S. S. Rhee, and E. Hunter, "Postassembly cleavage of a retroviral glycoprotein cytoplasmic domain removes a necessary incorporation signal and activates fusion activity," *Journal of Virology*, vol. 68, no. 7, pp. 4620–4627, 1994.

[72] M. A. Sommerfelt, S. R. Petteway Jr., G. B. Dreyer, and E. Hunter, "Effect of retroviral proteinase inhibitors on Mason-Pfizer monkey virus maturation and transmembrane glycoprotein cleavage," *Journal of Virology*, vol. 66, no. 7, pp. 4220–4227, 1992.

[73] N. R. Rice, L. E. Henderson, R. C. Sowder, T. D. Copeland, S. Oroszlan, and J. F. Edwards, "Synthesis and processing of the transmembrane envelope protein of equine infectious anemia virus," *Journal of Virology*, vol. 64, no. 8, pp. 3770–3778, 1990.

[74] E. Hunter, "Viral entry and receptors," in *Retroviruses*, J. M. Coffin, S. H. Hughes, and H. E. Varmus, Eds., pp. 71–119, Cold Spring Harbor Laboratory Press, Plainview, NY, USA, 1997.

[75] D. Fass, R. A. Davey, C. A. Hamson, P. S. Kim, J. M. Cunningham, and J. M. Berger, "Structure of a murine leukemia virus receptor-binding glycoprotein at 2.0 angstrom resolution," *Science*, vol. 277, no. 5332, pp. 1662–1666, 1997.

[76] D. Fass, S. C. Harrison, and P. S. Kim, "Retrovirus envelope domain at 1.7 Å resolution," *Nature Structural Biology*, vol. 3, no. 5, pp. 465–469, 1996.

[77] B. Kobe, R. L. Center, B. E. Kemp, and P. Poumbourios, "Crystal structure of human T cell leukemia virus type 1 gp21 ectodomain crystallized as a maltose-binding protein chimera reveals structural evolution of retroviral transmembrane proteins," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 8, pp. 4319–4324, 1999.

[78] A. Pinter, R. Kopelman, Z. Li, S. C. Kayman, and D. A. Sanders, "Localization of the labile disulfide bond between SU and TM of the murine leukemia virus envelope protein complex to a highly conserved CWLC motif in SU that resembles the active-site sequence of thiol-disulfide exchange enzymes," *Journal of Virology*, vol. 71, no. 10, pp. 8073–8077, 1997.

[79] E. O. Freed and R. Risser, "The role of envelope glycoprotein processing in murine leukemia virus infection," *Journal of Virology*, vol. 61, no. 9, pp. 2852–2856, 1987.

[80] J. A. Ragheb and W. F. Anderson, "pH-independent murine leukemia virus ecotropic envelope-mediated cell fusion: implications for the role of the R peptide and p12E TM in viral entry," *Journal of Virology*, vol. 68, no. 5, pp. 3220–3231, 1994.

[81] A. Rein, J. Mirro, J. G. Haynes, S. M. Ernst, and K. Nagashima, "Function of the cytoplasmic domain of a retroviral transmembrane protein: p15E-p2E cleavage activates the membrane fusion capability of the murine leukemia virus Env protein," *Journal of Virology*, vol. 68, no. 3, pp. 1773–1781, 1994.

[82] M. Li, Z. N. Li, Q. Yao, C. Yang, D. A. Steinhauer, and R. W. Compans, "Murine leukemia virus R peptide inhibits influenza virus hemagglutinin-induced membrane fusion," *Journal of Virology*, vol. 80, no. 12, pp. 6106–6114, 2006.

[83] M. Wallin, M. Ekström, and H. Garoff, "Isomerization of the intersubunit disulphide-bond in Env controls retrovirus fusion," *EMBO Journal*, vol. 23, no. 1, pp. 54–65, 2004.

[84] K. Li, S. Zhang, M. Kronqvist, M. Ekström, M. Wallin, and H. Garoff, "The conserved His8 of the Moloney murine leukemia virus Env SU subunit directs the activity of the SU-TM disulphide bond isomerase," *Virology*, vol. 361, no. 1, pp. 149–160, 2007.

[85] A. L. Barnett and J. M. Cunningham, "Receptor binding transforms the surface subunit of the mammalian C-type retrovirus envelope protein from an inhibitor to an activator of fusion," *Journal of Virology*, vol. 75, no. 19, pp. 9096–9105, 2001.

[86] A. L. Barnett, R. A. Davey, and J. M. Cunningham, "Modular organization of the Friend murine leukemia virus envelope protein underlies the mechanism of infection," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 7, pp. 4113–4118, 2001.

[87] D. Lavillette, A. Ruggieri, S. J. Russell, and F. L. Cosset, "Activation of a cell entry pathway common to type C mammalian retroviruses by soluble envelope fragments," *Journal of Virology*, vol. 74, no. 1, pp. 295–304, 2000.

[88] S. K. Chattopadhyay, M. R. Lander, E. Rands, S. Gupta and D. R. Lowy, "Origin of mink cytopathic focus-forming (MCF) viruses: comparison with ecotropic and xenotropic murine leukemia virus genomes," *Virology*, vol. 113, no. 2, pp. 465–483, 1981.

[89] J. W. Hartley, N. K. Wolford, L. J. Old, and W. P. Rowe, "A new class of murine leukemia virus associated with development of spontaneous lymphomas," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 74, no. 2, pp. 789–792, 1977.

[90] R. A. Bosselman, F. van Straaten, C. van Beveren, I. M. Verma, and M. Vogt, "Analysis of the env gene of a molecularly cloned and biologically active Moloney mink cell focus-forming proviral DNA," *Journal of Virology*, vol. 44, no. 1, pp. 19–31, 1982.

[91] A. Rein, "Interference grouping of murine leukemia viruses: a distinct receptor for the MCF-recombinant viruses in mouse cells," *Virology*, vol. 120, no. 1, pp. 251–257, 1982.

[92] D. L. Wensel, W. Li, and J. M. Cunningham, "A virus-virus interaction circumvents the virus receptor requirement for infection by pathogenic retroviruses," *Journal of Virology*, vol. 77, no. 6, pp. 3460–3469, 2003.

[93] G. J. Cianciolo, T. D. Copeland, S. Oroszlan, and R. Snyderman, "Inhibition of lymphocyte proliferation by a synthetic peptide homologous to retroviral envelope proteins," *Science*, vol. 230, no. 4724, pp. 453–455, 1985.

[94] G. Schlecht-Louf, M. Renard, M. Mangeney et al., "Retroviral infection in vivo requires an immune escape virulence factor encrypted in the envelope protein of oncoretroviruses," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 8, pp. 3782–3787, 2010.

[95] A. Rein and A. Schultz, "Different recombinant murine leukemia viruses use different cell surface receptors," *Virology*, vol. 136, no. 1, pp. 144–152, 1984.

[96] C. S. Tailor, D. Lavillette, M. Marin, and D. Kabat, "Cell surface receptors for gammaretroviruses," *Current Topics in Microbiology and Immunology*, vol. 281, pp. 29–106, 2003.

[97] H. M. Temin and H. Rubin, "Characteristics of an assay for Rous sarcoma virus and Rous sarcoma cells in tissue culture," *Virology*, vol. 6, no. 3, pp. 669–688, 1958.

[98] W. P. Rowe, W. E. Pugh, and J. W. Hartley, "Plaque assay techniques for murine leukemia viruses," *Virology*, vol. 42, no. 4, pp. 1136–1139, 1970.

[99] R. H. Bassin, N. Tuttle, and P. J. Fischinger, "Rapid cell culture assay technique for murine leukaemia viruses," *Nature*, vol. 229, no. 5286, pp. 564–566, 1971.

[100] S. J. Rulli Jr., J. Mirro, S. A. Hill et al., "Interactions of murine APOBEC3 and human APOBEC3G with murine leukemia viruses," *Journal of Virology*, vol. 82, no. 13, pp. 6566–6575, 2008.

[101] D. Mazurov, A. Ilinskaya, G. Heidecker, P. Lloyd, and D. Derse, "Quantitative comparison of HTLV-1 and HIV-1 cell-to-cell infection with new replication dependent vectors," *PLoS Pathogens*, vol. 6, no. 2, Article ID e1000788, 2010.

[102] R. H. Bassin, S. Ruscetti, I. Ali, D. K. Haapala, and A. Rein, "Normal DBA/2 mouse cells synthesize a glycoprotein which interferes with MCF virus infection," *Virology*, vol. 123, no. 1, pp. 139–151, 1982.

[103] H. Ikeda and T. Odaka, "A cell membrane "gp70" associated with Fv-4 gene: immunological characterization and tissue and strain distribution," *Virology*, vol. 133, no. 1, pp. 65–76, 1984.

[104] T. Pincus, W. P. Rowe, and F. Lilly, "A major genetic locus affecting resistance to infection with murine leukemia viruses. II. Apparent identity to a major locus described for resistance to friend murine leukemia virus," *Journal of Experimental Medicine*, vol. 133, no. 6, pp. 1234–1241, 1971.

[105] H. C. T. Groom, M. W. Yap, R. P. Galão, S. J. D. Neil, and K. N. Bishop, "Susceptibility of xenotropic murine leukemia virus-related virus (XMRV) to retroviral restriction factors," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 11, pp. 5166–5171, 2010.

[106] S. Best, P. L. Tissier, G. Towers, and J. P. Stoye, "Positional cloning of the mouse retrovirus restriction gene Fv1," *Nature*, vol. 382, no. 6594, pp. 826–829, 1996.

[107] M. W. Yap and J. P. Stoye, "Intracellular localisation of Fv1," *Virology*, vol. 307, no. 1, pp. 76–89, 2003.

[108] G. Duran-Troise, R. H. Bassin, A. Rein, and B. I. Gerwin, "Loss of Fv 1 restriction in Balb/3T3 cells following infection with a single N tropic murine leukemia virus particle," *Cell*, vol. 10, no. 3, pp. 479–488, 1977.

[109] R. H. Bassin, G. Duran-Troise, B. I. Gerwin, and A. Rein, "Abrogation of Fv-1(b) restriction with murine leukemia viruses inactivated by heat or by gamma irradiation," *Journal of Virology*, vol. 26, no. 2, pp. 306–315, 1978.

[110] K. N. Bishop, G. B. Mortuza, S. Howell, M. W. Yap, J. P. Stoye, and I. A. Taylor, "Characterization of an amino-terminal dimerization domain from retroviral restriction factor Fv1," *Journal of Virology*, vol. 80, no. 16, pp. 8225–8235, 2006.

[111] M. W. Yap, G. B. Mortuza, I. A. Taylor, and J. P. Stoye, "The design of artificial retroviral restriction factors," *Virology*, vol. 365, no. 2, pp. 302–314, 2007.

[112] L. Hilditch, R. Matadeen, D. C. Goldstone, P. B. Rosenthal, I. A. Taylor, and J. P. Stoye, "Ordered assembly of murine leukemia virus capsid protein on lipid nanotubes directs specific binding by the restriction factor, Fv1," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 108, no. 14, pp. 5771–5776, 2011.

[113] G. Towers, M. Bock, S. Martin, Y. Takeuchi, J. P. Stoye, and O. Danos, "A conserved mechanism of retrovirus restriction in mammals," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 22, pp. 12295–12299, 2000.

[114] R. S. Harris and M. T. Liddament, "Retroviral restriction by APOBEC proteins," *Nature Reviews Immunology*, vol. 4, no. 11, pp. 868–877, 2004.

[115] R. S. LaRue, V. Andrésdóttir, Y. Blanchard et al., "Guidelines for naming nonprimate APOBEC3 genes and proteins," *Journal of Virology*, vol. 83, no. 2, pp. 494–497, 2009.

[116] A. Low, C. M. Okeoma, N. Lovsin et al., "Enhanced replication and pathogenesis of Moloney murine leukemia virus in mice defective in the murine APOBEC3 gene," *Virology*, vol. 385, no. 2, pp. 455–463, 2009.

[117] C. M. Okeoma, N. Lovsin, B. M. Peterlin, and S. R. Ross, "APOBEC3 inhibits mouse mammary tumour virus replication in vivo," *Nature*, vol. 445, no. 7130, pp. 927–930, 2007.

[118] R. K. Holmes, M. H. Malim, and K. N. Bishop, "APOBEC-mediated viral restriction: not simply editing?" *Trends in Biochemical Sciences*, vol. 32, no. 3, pp. 118–128, 2007.

[119] J. L. Mbisa, R. Barr, J. A. Thomas et al., "Human immunodeficiency virus type 1 cDNAs produced in the presence of APOBEC3G exhibit defects in plus-strand DNA transfer and integration," *Journal of Virology*, vol. 81, no. 13, pp. 7099–7110, 2007.

[120] M. A. Langlois, K. Kemmerich, C. Rada, and M. S. Neuberger, "The AKV murine leukemia virus is restricted and hypermutated by mouse APOBEC3," *Journal of Virology*, vol. 83, no. 22, pp. 11550–11559, 2009.

[121] T. Paprotka, N. J. Venkatachari, C. Chaipan et al., "Inhibition of xenotropic murine leukemia virus-related virus by APOBEC3 proteins and antiviral drugs," *Journal of Virology*, vol. 84, no. 11, pp. 5719–5729, 2010.

[122] K. Stieler and N. Fischer, "Apobec 3G efficiently reduces infectivity of the human exogenous gammaretrovirus XMRV," *PLoS ONE*, vol. 5, no. 7, Article ID e11738, 2010.

[123] E. P. Browne and D. R. Littman, "Species-specific restriction of Apobec3-mediated hypermutation," *Journal of Virology*, vol. 82, no. 3, pp. 1305–1313, 2008.

[124] A. Abudu, A. Takaori-Kondo, T. Izumi et al., "Murine retrovirus escapes from murine APOBEC3 via two distinct novel mechanisms," *Current Biology*, vol. 16, no. 15, pp. 1565–1570, 2006.

[125] S. J. D. Neil, T. Zang, and P. D. Bieniasz, "Tetherin inhibits retrovirus release and is antagonized by HIV-1 Vpu," *Nature*, vol. 451, no. 7177, pp. 425–430, 2008.

[126] C. Goffinet, S. Schmidt, C. Kern, L. Oberbremer, and O. T. Keppler, "Endogenous CD317/tetherin limits replication of HIV-1 and murine leukemia virus in rodent cells and is resistant to antagonists from primate viruses," *Journal of Virology*, vol. 84, no. 21, pp. 11374–11384, 2010.

[127] D. T. Evans, R. Serra-Moreno, R. K. Singh, and J. C. Guatelli, "BST-2/tetherin: a new component of the innate immune response to enveloped viruses," *Trends in Microbiology*, vol. 18, no. 9, pp. 388–396, 2010.

[128] S. P. Goff, "Host factors exploited by retroviruses," *Nature Reviews Microbiology*, vol. 5, no. 4, pp. 253–263, 2007.

Hindawi Publishing Corporation
Advances in Virology
Volume 2011, Article ID 975801, 16 pages
doi:10.1155/2011/975801

Review Article

Naturally Occurring Polymorphisms of the Mouse Gammaretrovirus Receptors CAT-1 and XPR1 Alter Virus Tropism and Pathogenicity

Christine A. Kozak

Laboratory of Molecular Microbiology, National Institute of Allergy and Infectious Diseases, Bethesda, MD 20892-0460, USA

Correspondence should be addressed to Christine A. Kozak, ckozak@niaid.nih.gov

Received 5 May 2011; Accepted 12 July 2011

Academic Editor: Paul Jolicoeur

Gammaretroviruses of several different host range subgroups have been isolated from laboratory mice. The ecotropic viruses infect mouse cells and rely on the host CAT-1 receptor. The xenotropic/polytropic viruses, and the related human-derived XMRV, can infect cells of other mammalian species and use the XPR1 receptor for entry. The coevolution of these viruses and their receptors in infected mouse populations provides a good example of how genetic conflicts can drive diversifying selection. Genetic and epigenetic variations in the virus envelope glycoproteins can result in altered host range and pathogenicity, and changes in the virus binding sites of the receptors are responsible for host restrictions that reduce virus entry or block it altogether. These battleground regions are marked by mutational changes that have produced 2 functionally distinct variants of the CAT-1 receptor and 5 variants of the XPR1 receptor in mice, as well as a diverse set of infectious viruses, and several endogenous retroviruses coopted by the host to interfere with entry.

1. Introduction

The various inbred strains of laboratory mice and wild mouse species differ in their susceptibility to mouse gammaretrovirus infection and to virus-induced diseases. Host resistance is due to numerous constitutively expressed antiviral factors that target specific stages of the retroviral life cycle. These host restriction factors can block entry, postentry uncoating and reverse transcription, trafficking, integration, assembly, and release [1]. The first step in the replicative cycle is entry, and this process relies on host-encoded receptors. Host cell factors that can interfere with virus entry include genetic variations of the cell receptor as well as other host factors such as envelope (Env) glycoproteins produced by endogenous retroviruses (ERVs).

Infectious mouse leukemia viruses (MLVs) of three subgroups have been isolated from laboratory mice, and these subgroups were initially defined by their species tropisms. The ecotropic MLVs (E-MLVs) infect only mouse or rat cells and use the amino acid transporter CAT-1 as receptor. The xenotropic MLVs (X-MLVs) infect cells of non-rodent species [2], and polytropic MLVs (P-MLVs) infect both mouse and non-rodent cells [3, 4]. The X-MLVs and P-MLVs together constitute the XP-MLVs and both use the XPR1 receptor [5–8].

Receptor choice is determined by the N-terminal portion of the MLV Env, the receptor-binding domain (RBD) [9–11]. The E-MLVs and XP-MLVs both have Env subtypes that differ in their ability to use polymorphic variants of their cognate receptors, and some of these host-range variants, the "xenotropic" MLVs, are completely restricted in mouse cells [12]. Both receptors for the laboratory mouse MLVs, CAT-1 and XPR1, have naturally occurring variants responsible for specific virus resistance phenotypes. There are 2 functionally distinct variants of the CAT-1 receptor for E-MLVs [13], and there are 5 known variants of the XPR1 receptor for the XP-MLVs in mice [5, 14–17]. These variants are not only important host factors that can restrict infection, but also they can alter virus-receptor interactions in ways that influence virus-induced pathology. This paper

will describe the functional variants of these 2 MLV receptors and describe their coevolution with MLV in virus-infected mouse populations.

2. The CAT-1 Receptor for E-MLVs

The first gammaretrovirus receptor gene to be cloned was the CAT-1 receptor for E-MLVs [18]. This gene (gene symbol *Slc7a1*) encodes a glycoprotein with 14 putative transmembrane domains, and it functions as a cationic amino acid transporter [19, 20] (Figure 1(a)). Ten additional gammaretrovirus receptors have now been cloned; all of these gammaretrovirus receptors are multi-transmembrane proteins, and the receptors with known functions are all transporters of small solutes (reviewed in [21–26]). The human orthologue of mouse CAT-1 does not function as an E-MLV receptor, and the key sites in the mouse protein critical for virus entry lie in the third extracellular loop along with two consensus recognition sites for N-linked glycosylation [27, 28] (Figures 1(a) and 1(b)). CAT-1 is modified posttranslationally by glycosylation, and N-glycans are added to both of the CAT-1 loop 3 glycosylation sites [29]. All E-MLVs rely on the CAT-1 receptor for entry, although initial binding and the efficiency of entry may be influenced by other factors at the cell surface, such as heparin [30, 31].

The E-MLV Env glycoprotein consists of surface (SU) and transmembrane (TM) subunits that are proteolytically cleaved from the same precursor protein and are linked by disulfide bonds. The SU protein has a 236 residue RBD at its N-terminal end that has 3 variable regions, and this is followed by a proline-rich hinge region and a C-terminal domain (Figure 2(a)). Entry is initiated by virus-receptor binding which precipitates a conformational change in Env that allows for subsequent fusion of viral and cellular membranes, a process that may involve cellular proteases [32]. The CAT-1 receptor recognition site is within the first variable domain (VRA) of the RBD. Three residues within the RBD VRA of the Friend E-MLV, FrMLV, have been identified as critical for entry (S84, D86, and W102) [33–35]. The crystal structure and functional analysis of the FrMLV RBD showed that these residues form a binding pocket on the structure's surface [35, 36] (Figure 2(b)). Several additional Env residues outside the binding pocket affect postbinding entry. Thus, the H8 residue in a conserved SPHQV motif near the SU N-terminus is necessary for fusion [37, 38] although residues at the other end of the MoMLV RBD, at positions 227 and 243 (equivalent to FrMLV sites 229 and 245), can substitute for H8 [39]. The proline-rich region is also involved in mediating postbinding conformational changes and fusion [40], and residues in two segments of the C-terminus of Env also have roles in fusion [41–43] (Figure 2(a)).

3. Variants of the CAT-1 Receptor and E-MLV Env Affect Virus Entry

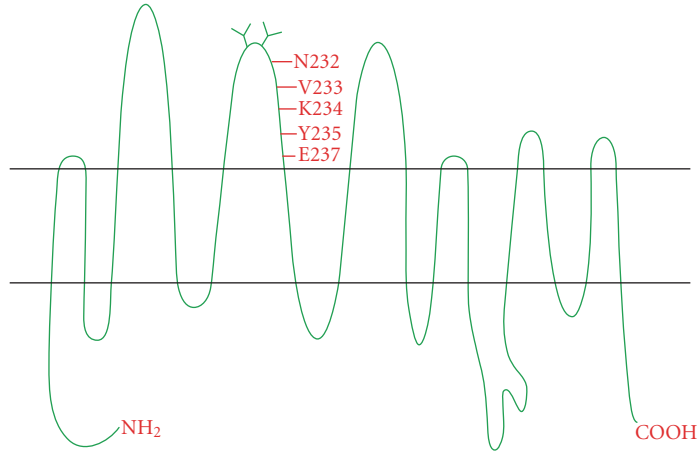
There has been no systematic attempt to screen for CAT-1 receptor variation in mice, but 3 sequence variants have been identified in *Mus* (Figure 1(b)). The prototype receptor, mCAT-1, was cloned from NIH 3T3 cells [18]. Two sequence variants have been identified in the wild mouse species *M. dunni* and *M. minutoides* [13, 44]. Limited testing suggests that the *M. minutoides* CAT-1 functions like the laboratory mouse mCAT-1 receptor, but the receptor of *M. dunni*, dCAT-1, differs from mCAT-1. *M. dunni* cells are relatively resistant to infection by Moloney E-MLV (MoMLV), although these cells are fully susceptible to other E-MLV isolates [13]. dCAT-1 differs from mCAT-1 by 4 residues, two of which are in the receptor determining third extracellular loop; one, I214V, is a substitution, and the second is a glycine insertion within the NVKYGE virus binding site [13] (Figure 1(b)).

Two mutational changes in the MoMLV RBD VRA independently produce viruses that efficiently infect *M. dunni* cells: a replacement mutation, S82F, and introduction of two serine residues that are present in other E-MLV VRAs but absent in MoMLV (S76, S77 in FrMLV) (Figure 2(a)) [45]. The MoMLV S82F mutation site corresponds to S84 in Friend MLV, one of the 3 residues critical for virus binding and entry. The importance of this residue for virus tropism is underscored by the fact that MoMLV-S82F is poorly infectious in cells that carry mCAT-1 [45] (Figure 1(b)).

E-MLVs can infect rodent species in addition to *Mus*, and CAT-1 receptor variants have been described in hamsters and rats (Figure 1(b)). Hamster cells are generally resistant to infection by E-MLVs, but some variants of FrMLV can infect these cells [46]. Infectivity of one such variant, PVC-211, was attributed to Env substitutions E116G and E129K [47] (Figure 2). Another FrMLV variant, F-S MLV, also inefficiently but reproducibly infects hamster cells; it was suggested that this tropism was influenced by two substitutions: S84A and S79N [48]. Restriction of some E-MLVs can result from complementary changes in the interacting sites of virus Env and the CAT-1 receptor; however, the MoMLV restriction associated with dCAT-1 is reproduced in human cells expressing this receptor [13], but not in ferret cells [49], suggesting that other cellular factors may also influence receptor function.

4. CAT-1 and Env Polymorphisms Associated with Pathogenicity

Polymorphisms that alter virus-receptor interactions can affect pathogenesis as well as entry. Cytopathic variants are common among the retroviruses that induce disease in their hosts, including HIV-1 as well as avian leukosis viruses and some pathogenic bovine and feline leukemia viruses [50–52]. These viruses can produce large multinucleated syncytia in cultures of susceptible cells. In contrast, mouse gammaretroviruses rarely produce syncytia although there are three exceptional cytopathic E-MLVs. The MoMLV variant, Spl574 and a FrMLV variant, F-S MLV, both induce syncytia and cell death in *M. dunni* cells [45, 48]. The third cytopathic virus, TR1.3, is a neuropathic FrMLV variant that also induces syncytia in SC-1 cells [33].



(a)

		LOG10 VIRUS INFECTIVITY		
		MoMLV	MoMLV-S82F	AKV MLV
mCAT-1	VKGSIKNWQLTEK---NFS---CNNNDTNVKY-GEGGFM	5	3	4
dCAT-1	....V.....---.....---....G.....	2	5	4
minCAT-1	.....Q...---.....---.L.....	ND	ND	4
haCAT-1	.....EDFL.R.SPL.G.---...H.....			
rCAT-1	.....E.....E....K.SPL.G.---.....			
xcCAT-1	.....E.....E....K.SPL.G.D.....			
huCAT-1	....V.....EDFG.T.GRL.L.---KEGKP.V....			

(b)

Figure 1: Predicted topology and sequence variation of the CAT-1 receptor for mouse ecotropic gammaretroviruses. (a) Putative topology identifies 14 predicted transmembrane domains. The third extracellular loop contains critical residues for receptor function (in red) and two N-linked glycosylation sites. (b) Sequence variation in the CAT-1 third extracellular loop. At the top are three sequence variants found in *Mus* with residues critical for entry in red. Virus infectivity of cells expressing these receptors is measured as the $\log_{10}$ titer of FFU/100 μL of viral Env pseudotypes carrying the LacZ reporter gene; ND: not done. Consensus sites for N-glycosylation are underlined. CAT-1 sequence variation is shown for mouse CAT-1 variants mCAT-1 (NIH 3T3), dCAT-1 (*M. dunni*), and minCAT-1 (*M. minutoides*). E-MLV-infected *Mus* species *M. castaneus*, *M. molossinus*, *M. spicilegus*, and *M. musculus* are identical to mCAT-1 in the indicated region. Also shown are CAT-1 sequences for virus-susceptible species hamster (ha), rat (r), and XC rat cells (xc) and for virus-resistant human (hu).

The cytopathicity of these 3 viruses is due to single amino acid substitutions at two of the 3 amino acids that form the receptor binding site. The cytopathicity of TR1.3 is due to W102G [33], and the cytopathicity of the other variants is due to different amino acid substitutions at the same critical Env residue: S82F in Spl574, and S84A in F-S MLV [45, 48]. Syncytium formation by Spl574 and F-S MLV is accompanied by the accumulation of large amounts of unintegrated viral DNA [48], a phenomenon which is also a hallmark of other cytopathic retroviruses and has been attributed to the absence of superinfection interference [53]. TR1.3 shows significantly reduced receptor binding avidity that correlates with its inability to block superinfection [54]. That cytopathicity is a consequence of altered receptor virus interactions is also supported by the fact, noted above, that MoMLV-S82F shows altered host range (Figure 1(b)) and also by the fact that syncytia formation by Spl574 is observed in cells of heterologous species expressing dCAT-1, but not mCAT-1 [49]. Thus, the cytopathicity of these 3 viruses in cultured cells and the neurovirulence of TR1.3 are governed by sequence differences in the viral *env* and, for 2 of these viruses, by corresponding differences in the CAT-1 receptor.

Other polymorphisms of the E-MLV *env* can alter cell tropism and influence disease type. The thymotropism of radiation leukemia virus has been mapped to *env* [55], and several E-MLVs have neuropathogenic properties due to *env* polymorphisms. For example, TRM, a mutant variant of the neuropathic TR1.3, induces a different disease pathology resulting from reversion of the TR1.3 G102W mutation and a new Env mutation, S159P [56]. The most extensively studied neuropathic E-MLV is CasBrE, an isolate from California wild mice. Early studies mapped neurovirulence determinants to the CasBrE Env [57], and recent data indicate that CasBrE neuropathology is mediated by Env at two levels. First, the CasBrE Env targets the virus to cells within the CNS that express significant levels of CAT-1, and second, disease-associated spongiosis is induced by MLV-receptor-independent toxicity of this Env, determinants of which

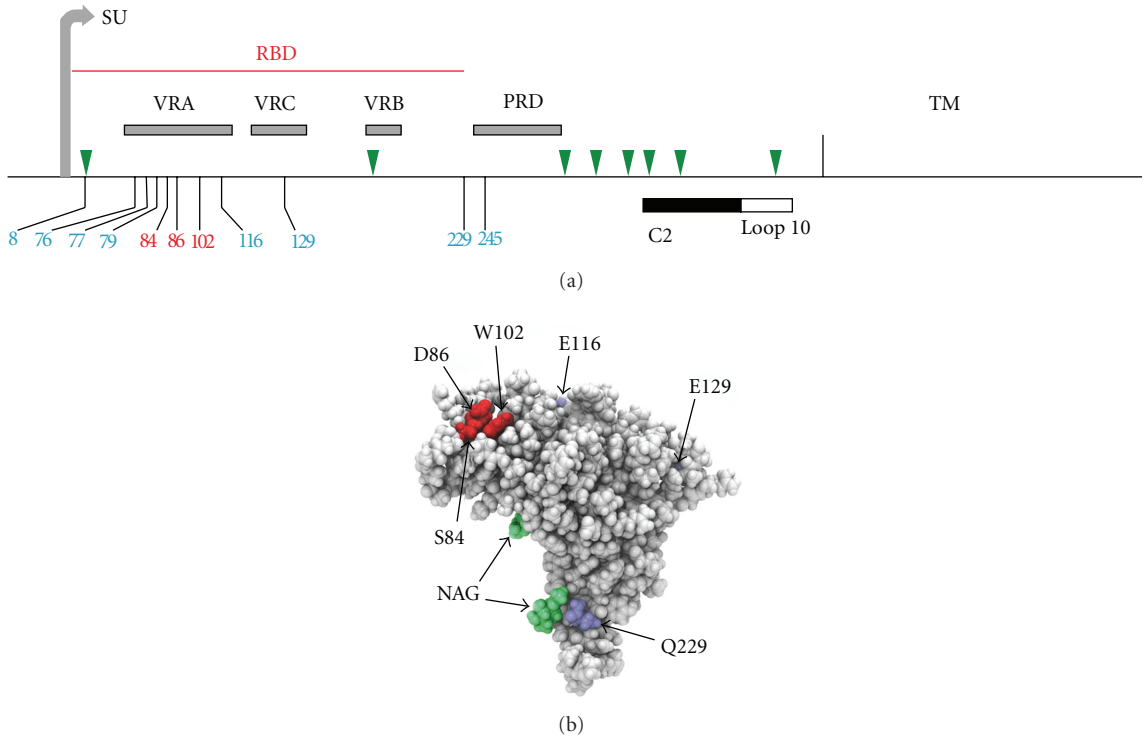


Figure 2: Structure of the FrMLV E-MLV Env gene. (a) Stick figure representation identifies the surface (SU) and transmembrane (TM) domains, the receptor binding domain (RBD) containing three variable regions (VRA, VRB, and VRC), and the proline-rich domain (PRD). Green triangles mark the N-linked glycosylation sites in SU. Vertical lines identify residues with roles in entry; the three residues in red form the binding pocket. The C-terminal segments designated C2 and loop10 have also been implicated in entry [42, 43]. (b) Surface representation of the FrMLV RBD (PDB ID 1AOL) [36], showing the location of the binding pocket (red), additional residues involved in entry (blue), and two N-linked glycosylation sites (green).

have not been identified but are presumably shared with other neurovirulent MLVs [58]. Env residues have also been implicated in the targeting of the neuropathic Friend PVC-211 variant to brain capillary endothelial cells [31]. This tropism is due to 2 mutations, E116G in VRA and E129K in VRC, mutations that also alter host range and interference properties [47, 59]. These findings indicate that specific replacement substitutions at different positions in the Env of pathogenic E-MLVs can affect receptor interactions, cell tropism, disease induction, and disease type.

5. The Role of Glycosylation in E-MLV Entry and Tropism

The retroviral Env is glycosylated, as are cellular proteins involved in entry. Many viruses use the glycans on cell surface glycoproteins as attachment factors [60], but glycosylation of the CAT-1 receptor is not required for virus entry. CAT-1 continues to support virus entry after both loop 3 N-glycan sites have been removed by mutagenesis [61]. However, host cell glycans can modulate entry of some E-MLVs. Thus, resistance of *M. dunni* cells to MoMLV, resistance of NIH 3T3 cells to Spl574, and resistance of primary rat fibroblasts and hamster cells to E-MLVs are relieved by inhibitors of glycosylation [49, 62–66]. It is not clear whether the responsible glycoprotein is CAT-1 or other host glycoproteins, like the secreted factor associated with resistance to gibbon ape leukemia virus in hamster cells [64]. There is, however, some evidence that the restriction of E-MLV infection in rat cells may be regulated by the glycosylation of rat CAT-1. The CAT-1 of rat XC sarcoma cells lacks one of the glycosylation sites found in the CAT-1 gene of other rat cells (Figure 1(b)), and heterologous cells expressing xcCAT-1 were found to be more susceptible to MoMLV than cells expressing rCAT-1 [67].

Glycosylation of the viral Env has been associated with altered infectivity of multiple viruses including retroviruses such as HIV-1 [68]. MLV Envs can have up to 9 N-linked glycans (Figure 2(a)), and while glycans are critical for the maturation and transport of Env [69], functional roles for the individual Env glycans are poorly defined. It has been shown that loss of MoMLV gs2 results in a virus that is temperature sensitive in Rat2 cells, loss of gs4 produces noninfectious virus lacking SU protein, and loss of gs7 alters fusion and infectivity [70–73]. Removal of either of the 2 glycosylation sites in the Env RBD, gs1 and gs2, can produce viruses restricted by *M. dunni* cells due to altered virus binding to dCAT-1, although E-MLVs differ in their reliance on these glycans [74, 75]. Thus, N-linked glycans on the viral Env are required for proper folding and can influence the entry process, while glycans are not needed for CAT-1

receptor processing or receptor function and have, at best, limited ability to modulate virus entry.

6. CAT-1 and E-MLV Env Variation in Wild Mouse Species

Exposure to E-MLV gammaretroviruses occurred only recently in the evolution of *Mus* [76]. Although E-MLV ERVs are found in few of the 40 *Mus* species, wild mice carry three distinctive Env subtypes of E-MLVs (Figure 3). Sequence identity in SU*env* among these virus types is 70–77%. The first E-MLV type, the AKV E-MLVs of the laboratory mouse, is found as ERVs in multiple inbred strains [77]. Many of these proviruses are capable of producing infectious virus [78], and the widely used laboratory virus strains MoMLV, FrMLV, and Rauscher MLV are derived from AKV MLV [79] (Figure 3). Among the wild mouse species, AKV MLV ERVs are found in the Asian species *M. molossinus* and in *M. musculus* of Korea and China but not eastern Europe [76, 80]. A second E-MLV subtype was initially identified in California wild mice [81, 82]. Proviruses with this CasBrE Env type have also been found in the Asian species *M. castaneus,* and these virus-infected mice were likely introduced to California by passive transport from Asia [76, 80, 83, 84]. A third E-MLV subtype, HoMLV, was isolated from the eastern European species *M. spicilegus*, but is transmitted only as an exogenous virus [85].

The evidence indicates that these 3 E-MLV Env variants did not coevolve with receptor polymorphisms. Sequence comparisons indicate that the 3rd extracellular loop of the CAT-1 gene is invariant in wild-derived mice carrying these 3 E-MLVs: *M. castaneus*, *M. molossinus*, *M. spicilegus,* and *M. musculus* (GenBank Accession nos. JN226407–JN226410). The only known functional variant of this receptor in mice is dCAT-1 of *M. dunni*, and it is not clear if this variant arose in mice exposed to virus; the single available *M. dunni* sample is a cell line that does not carry E-MLV ERVs indicative of past infections [86], and it has not been determined if dCAT-1 is present in natural populations of *M. dunni* (now termed *M. terricolor*) or whether it originated by mutation in this cultured cell line. Sequence conservation of the receptor determining region of CAT-1 in *Mus* is not due to functional constraints as the third extracellular loops of the CAT-1 (SLC7A1) genes of various non-*Mus* species are quite variable (Figure 1(b)). Although E-MLVs can use CAT-1 receptor variants in non-*Mus* rodents, the replacement mutations in other mammalian species are incompatible with receptor function, thus limiting E-MLVs to rodents, a type of host range restriction that is not shared by other gammaretroviruses. The absence of polymorphism in the *Mus* CAT-1 gene, even in virus-infected wild mouse populations, is consistent with the conclusion that exposure to E-MLVs is very recent in *Mus* [76] and suggests that these mice rely on alternative survival strategies to limit the deleterious effects of infection, including posttranslational modification of receptor function, receptor interference, or postentry blocks in the retroviral lifecycle.

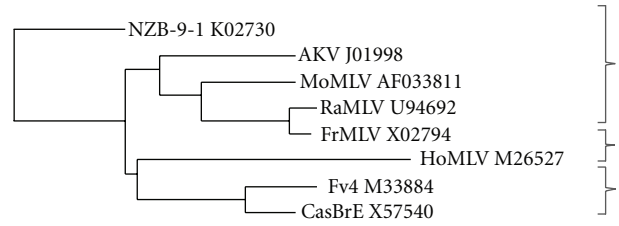


Figure 3: Phylogenetic tree of the Env genes of E-MLV gammaretroviruses. The tree includes laboratory mouse isolates FrMLV, MoMLV, and Rauscher MLV (RaMLV), the naturally occurring viruses AKV MLV, CasBrE, and HoMLV, and the Env gene of the *Fv4* restriction gene. The three related groups are bracketed. Sequences from GenBank were aligned using ClustalW2 and used to generate neighbour-joining trees. The X-MLV NZB-9-1 was included to root the tree.

7. The XPR1 Receptor for XP-MLVs

Two subgroups of nonecotropic MLVs have been isolated from laboratory mice. These viruses were originally described as having distinct host ranges, but they use the same receptor, XPR1. X-MLVs and P-MLVs are both capable of infecting cells of nonrodent species, and although P-MLVs can efficiently infect mouse cells, X-MLVs were initially identified as incapable of infecting their natural hosts [2, 87, 88]. X-MLVs and P-MLVs are closely related viruses, and sequence differences in *env* and LTR are responsible for their differences in species tropism, for their nonreciprocal interference patterns, and for the pathogenicity of P-MLVs in mice [11, 89–92]. Although it is clear that the RBD VRA region is the major determinant of P-MLV and X-MLV host range [11], the critical VRA residues involved in XPR1 receptor recognition have not been identified, although 2 residues outside VRA can influence the ability of these viruses to infect cells of other mammalian species (Figure 4) [93]. Viruses in the XP-MLV family are highly variable in the Env segment containing the RBD (Figure 4), and the wild mouse viruses, CasE#1 and Cz524, show atypical host range patterns that distinguish them from prototypical P-MLVs and X-MLVs (Table 1) [16, 89, 94].

The XPR1 receptor was originally described in laboratory mice as a P-MLV susceptibility gene [14]. Subsequent studies demonstrated that wild-mouse-derived cell lines, SC-1 and *M. dunni*, are susceptible to X-MLVs as well as P-MLVs [86, 96], while cells of the Asian species *M. castaneus* are resistant to P-MLVs [15]. That a single gene controls susceptibility to these two viruses was supported by the equivalent chromosome map locations of the genes controlling wild mouse susceptibility to X-MLVs and P-MLVs and by their cross-interference [5, 15, 89, 97]. The human and mouse *Xpr1* genes were cloned [6–8] and shown to encode a protein with 8 putative transmembrane domains (Figure 5). While a cellular function has not been assigned to XPR1, XPR1 is upregulated following activation of the NF-κB RANKL-RANK signaling pathway [98] and its closest homologues in yeast (SYG1) and plants (PHO1) function

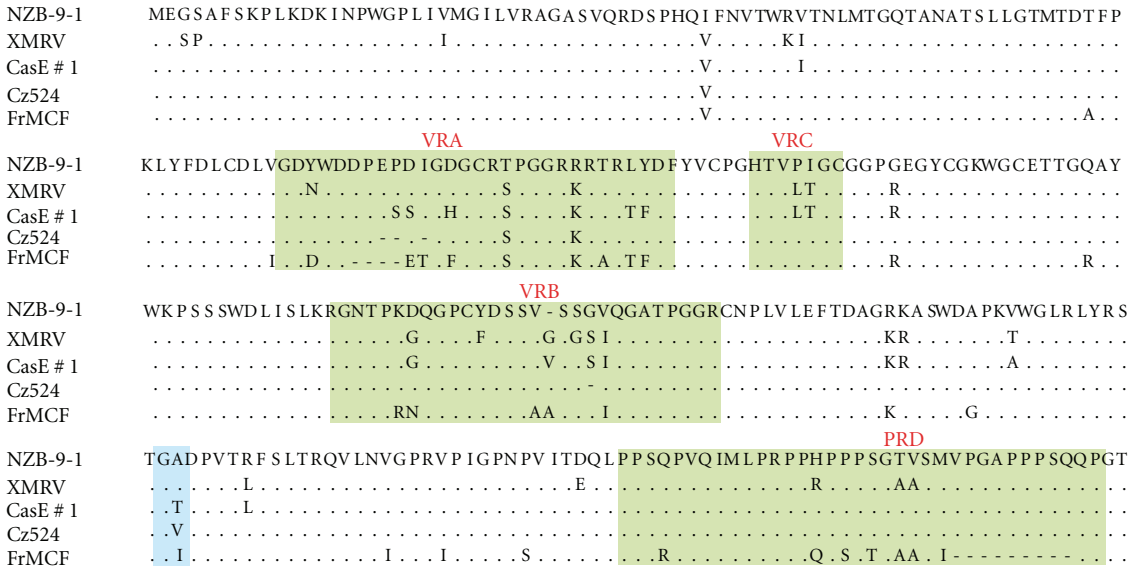


Figure 4: Alignment of the predicted amino acid sequences of the N-terminal portion of the Env sequences of various XP-MLVs. Included are the prototype NZB-9-1 X-MLV, the Friend FrMCF P-MLV, the wild mouse isolates CasE#1 and Cz524, and XMRV. Green blocks identify the three variable domains of the RBD and the PRD, and a blue block identifies two residues that influence species tropism [93].

Table 1: Functional variants of the XPR1 receptor in *Mus*.

	Infectivity[a]				Distribution[b]	
	X-MLV	P-MLV	CasE#1	Cz524	Common laboratory strains	*Mus* Species
Xpr1^{n}	−	+++	−	−	Most	None
Xpr1^{sxv}	+++	+++	+++	+++	F/St, LT, LP, SWR, SJL, SIM.R, SOD1	Most
Xpr1^{p}	+++	−	+++	−	None	*M. pahari*
Xpr1^{c}	++	−	−	+	None	*M. castaneus*
Xpr1^{m}	++	−	−	−	None	*M. musculus*, *M. molossinus*

[a] Measured as $\log_{10}$ titer of FFU/100 μL of viral Env pseudotypes carrying the LacZ reporter. $\log_{10}$ titer: +++, >3; ++, 2-3; +, 1-2; −, 0-1.
[b] Determined for ~50 of the common strains of laboratory mice and ~20 of the 40 species of *Mus* [17, 95].

in signal transduction and phosphate sensing and transport, respectively [8].

8. Naturally Occurring Variants of the XPR1 Receptor in *Mus*

The genus *Mus* includes about 40 species, and all available species have been screened for sequence and functional variants of *Xpr1* (Figures 5 and 6). Of the 5 sequence variants found in wild mice, 4 show unique receptor phenotypes based on their ability to support entry of different virus isolates that rely on this receptor (Table 1). The most common receptor variant among wild mouse species was originally termed *Sxv* (susceptibility to xenotropic virus) [5]. This variant is found in many Asian species as well as western European house mice [17, 99], and mice with *Sxv* were introduced into the Americas by European immigrants and explorers (Figure 7). *Sxv* is also carried by several of the common inbred strains of laboratory mice [95]. *Sxv* is the most permissive of the *Xpr1* alleles and supports entry of all XP-MLV host range variants (Table 1). The second most geographically widespread *Xpr1* allele, *Xpr1*^{m}, is found in two house mouse species, *M. musculus*, which ranges from central Europe to the Pacific, and *M. molossinus*, found in Japan [17]. This variant is highly restrictive, allowing inefficient entry of X-MLVs, while restricting all other XP-MLVs. A third allele, *Xpr1*^{c}, is found in the southeast Asian mouse, *M. castaneus*, and is responsible for resistance to infection by P-MLVs [15, 99]. A fourth wild mouse *Xpr1* allele is restricted to the Asian species *M. pahari*; these mice are susceptible to X-MLVs and to CasE#1 [16] (Table 1).

There is a fifth mouse *Xpr1* variant, *Xpr1*^{n}. The first of the receptor alleles to be identified, *Xpr1*^{n}, was cloned

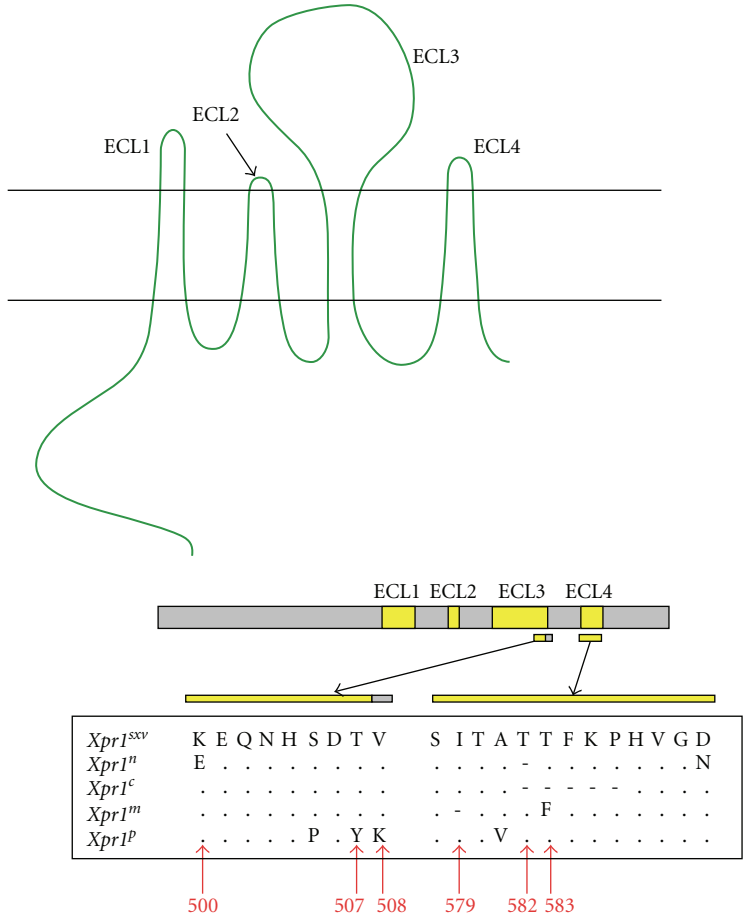


Figure 5: Predicted topology and sequence variation of the XPR1 receptor for XP-MLVs. At the top is shown the predicted structure with eight putative transmembrane domains and 4 extracellular loops (ECLs). The center diagram shows the relative locations of the 4 ECLs in the XPR1 protein, and the bottom shows sequence variation in the two ECLs involved in virus entry. Sequence is provided for the 5 functional XPR1 variants in *Mus*, and the red arrows indicate the 6 residues involved in entry.

from NIH 3T3 laboratory mouse cells [6–8]. This variant is responsible for the restrictive phenotype originally used to define the "xenotropic" host range subgroup, that is, viruses unable to infect cells of their home species [12]. *Xpr1*^{n} is unusable by X-MLVs or by the 2 wild mouse virus isolates, although it supports entry by P-MLVs (Table 1). The origin of this laboratory mouse variant is unclear. Although *Xpr1*^{n} is carried by the majority of common inbred mouse strains, it has not been found in any wild-trapped mouse [17, 95] (Figure 6). The common strains of laboratory mice were derived from colonies of fancy mice maintained by hobbyists, and they represent a mosaic of wild mouse species [100, 101]. Genomic analysis of multiple strains indicates that the predominant contributor to the laboratory mouse is *M. domesticus*, the western European mouse, with smaller contributions from *M. musculus* and *M. castaneus* [101]. Although this would suggest an *M. domesticus* origin for the laboratory mouse receptor allele, *M. domesticus* mice trapped in disparate locations in Europe and the Americans all carry the permissive allele [17] suggesting that *Xpr1*^{n} arose and/or was selected in the fancy mice (Figure 7).

9. Genetic Basis of XPR1 Functional Polymorphism

Initial studies on *Xpr1* receptor function focused on sequence differences between the phenotypic variants identified in NIH 3T3 cells (*Xpr1*^{n}) and *M. dunni* (*Xpr1*^{sxv}) [99]. Two critical amino acids were identified for X-MLV entry that lie in different putative extracellular loops (Figure 5). The restrictive *Xpr1*^{n} carries a substitution, K500E, in its third extracellular loop (ECL3), and a deletion, T582Δ, in the fourth loop (ECL4). Corrective mutations at either of these sites produce functional receptors for X-MLVs without compromising P-MLV receptor function [99]. Subsequent studies on the mouse receptor showed that these 2 critical residues are not equivalently used by the XP-MLVs, as CasE#1 can use *Xpr1*^{n}-Δ582T but not *Xpr1*^{n}-E500K [16]. Mutational analysis of other polymorphic sites in the various *Mus Xpr1s* identified residues at additional sites that modulate virus entry: ECL3 positions 500, 507, and 508 and ECL4 positions 579 and 583 (Figure 5) [17, 94].

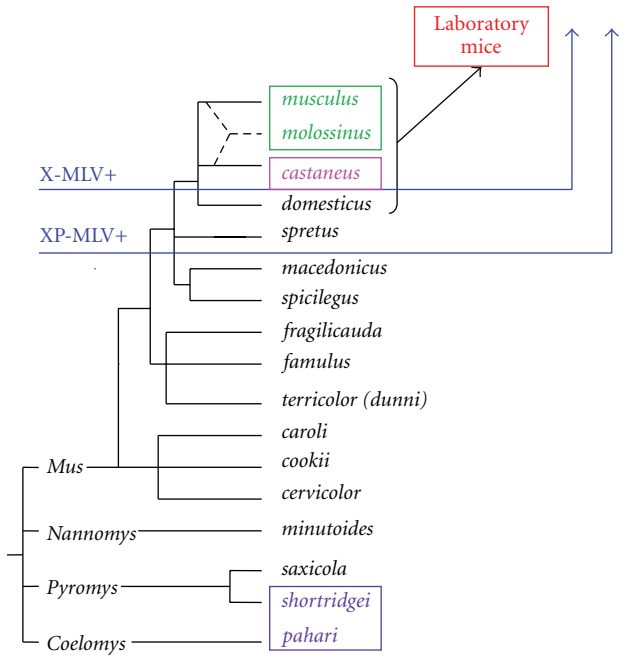


FIGURE 6: Phylogenetic tree of *Mus*. Blue arrows indicate the species that have acquired XP-MLV ERVs, and the subset that have predominantly X-MLVs. 4 colored boxes identify the mice carrying the 4 restrictive *Xpr1* alleles; all other species carry the permissive $Xpr1^{sxv}$.

Although it is possible that the XPR1 protein carries two separate receptor determinants on ECL3 and ECL4 [102], it is more likely that the key residues in ECL3 and ECL4 form a single virus attachment site. The various viruses that use XPR1 for entry are sensitive to mutational changes in both ECL3 and ECL4 [16, 17, 94] (Figure 8). Thus, P-MLVs and the wild mouse viruses CasE#1 and Cz524 show different patterns of infectivity for $Xpr1^{p}$ mutants that have substitutions in ECL3 but identical ECL4 sequences. These same viruses also differ in their infectivity for cells with $Xpr1^{m}$, $Xpr1^{c}$, and $Xpr1^{sxv}$, which have identical ECL3 sequences but different deletions in ECL4. The involvement of residues in multiple receptor domains is also characteristic of other retrovirus receptors [103]. While the different domains required for these other retroviral receptors can have distinctive roles in virus attachment and entry [104, 105], this division of labor has not yet been shown to be the case for the XPR1 ECL3 and ECL4 domains.

10. P-MLV Entry That Is Independent of the XPR1 Receptor

Although it is clear that MLV entry is typically mediated by specific cell surface receptors, some MLVs are capable of bypassing the need for their cognate receptors and can infect cells that lack receptors and may also be able to infect cells in which those receptors are downregulated by superinfection [106, 107]. Such alternative entry mechanisms seem to be particularly important for P-MLVs, viruses that are less able to establish effective superinfection immunity against further infection [99, 108] either because they may have lower binding affinity for the XPR1 receptor than the X-MLVs or because Env-bound receptors may recycle rapidly into acidic compartments where the Env-receptor complex is disrupted allowing the freed receptor to recycle back to the plasma membrane. This ineffective or delayed establishment of interference to exogenous infection has been linked to the massive accumulation of viral DNA in P-MLV-infected mice [109] and to the ability of P-MLVs to induce cytopathic responses in mink lung cells in which superinfection induces an ER stress response and apoptosis [4, 110, 111].

Infectious P-MLVs arise in preleukemic tissues of mice with high levels of E-MLVs. P-MLVs have recombinant genomes in which *env* sequences are derived from endogenous polytropic or modified polytropic sequences, the *Pmvs* or *Mpmvs* [112]. The *Pmv* and *Mpmv* proviruses that give rise to these recombinants have not been shown to be capable of producing infectious virus directly [113], but transmission of these ERVs and recombinant infectious P-MLVs can be accomplished through several mechanisms that are independent of XPR1. First, P-MLVs are generally transmitted in viremic mice as pseudotypes of E-MLVs and thus use mCAT-1 for entry [114, 115]. Also, homodimers representing the transcribed products of *Mpmv* and *Pmv* proviruses can be packaged into E-MLV virions, and these "mobilized" proviruses can infect cells, replicate in those new cells, and spread to other cells as pseudotyped virus [116]. Another transmission mechanism allows infectious, recombinant P-MLVs to use alternative receptors in the presence of the soluble RBD glycoprotein for that receptor. P-MLVs and entry defective E-MLVs, but not X-MLVs, can be "transactivated" in this way by E-MLV RBD [38, 108]. This transactivation process is controlled, at least in part, by the conserved Env residue H8 and has also been described for other viruses such as feline leukemia viruses [117]. While these mechanisms provide alternative routes for P-MLV transmission that are independent of the receptor, it should be noted that interference with the XPR1 receptor protects mice from P-MLV-induced disease [118] highlighting the crucial role of XPR1-mediated entry in the disease process.

11. Coevolution of XPR1 and XP-MLVs in *Mus*

The species distribution of the *Mus* XPR1 variants indicates that polymorphic, virus-restrictive receptors appeared when mice were exposed to XP-MLVs, especially X-MLVs (Figure 6). For most of the 8 million years of *Mus* evolution, species carried the permissive $Xpr1^{sxv}$ allele. Mice were subjected to XP-MLV infection about 0.5 MYA, and this exposure is marked by the acquisition of MLV ERVs in the 4 house mouse species [76, 119, 120]. *M. domesticus* carries P-MLV ERVs, whereas *M. castaneus*, *M. musculus,* and *M. molossinus* carry predominantly X-MLVs [76]. The common laboratory mouse strains, which are mosaics of these wild mouse species, generally carry multiple copies of X-MLVs

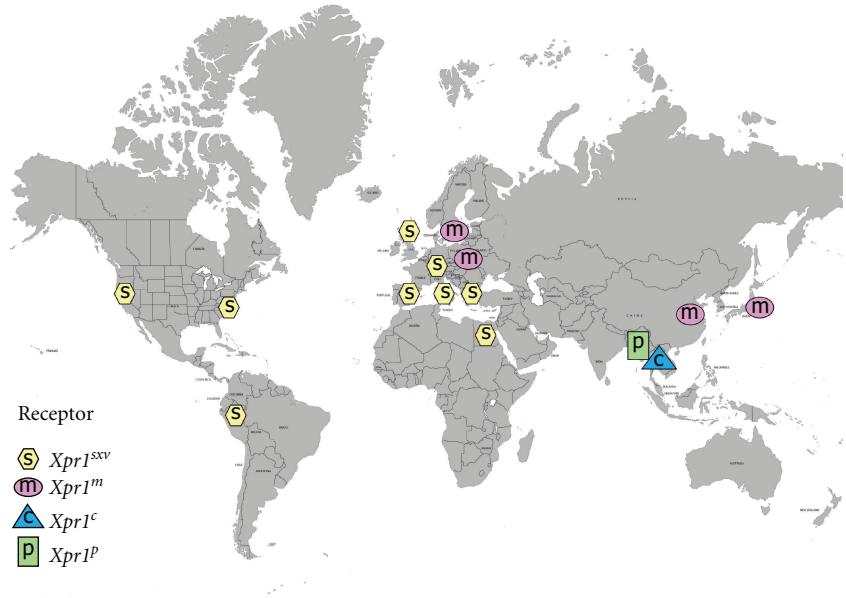


Figure 7: Geographic distribution of *Xpr1* alleles in wild mouse populations.

	ECL3	ECL4	X-MLV	P-MLV	Cz524	CasE # 1
M. pahari	THKEQNHPDYK	SITVTTFKPHVGD	++	–	–	++
Xpr1^{p}-ESTV	. . E . . . . S . TV	. . . . .	++	++	+	+
Xpr1^{p}-Y507T	. . . . . T .	. . . . .	++	–	++	++
M. dunni	THKEQNHSDTV	SITATTFKPHVGD	++	++	++	++
M. castaneus	. . . . .	. . . . - - - - - - - . . .	++	–	+	–
M. musculus	. . . . .	. - . . . K	+	–	–	–

Figure 8: The entry of various XP-MLVs is affected by sequence variation in ECL3 or ECL4 suggesting that these domains form a single receptor site.

and P-MLVs [121, 122]. The acquisition of these germline ERVs, and specifically X-MLVs, roughly coincides with the appearance of restrictive *Xpr1* alleles: *Xpr1*^{c} in *M. castaneus*, *Xpr1*^{m} in *M. molossinus* and *M. musculus*, and *Xpr1*^{n} in laboratory mice (Figure 6). Each of these restrictive receptors carries a unique deletion in the XPR1 ECL4 (Figure 5) [17]. *M. domesticus*, the species carrying only inactive P-MLV ERVs, maintains the full-length, permissive *Xpr1*^{sxv} receptor common to ancestral species of *Mus*. The fact that restrictive receptors have evolved in X-MLV infected mice suggests that this host pathogen interface has been an important evolutionary battleground. This also suggests that X-MLV infection is deleterious for mice, although the consequences of X-MLV infection have not yet been described because mouse gammaretroviruses have been studied largely in X-MLV-resistant laboratory mice. The discovery of mouse species and strains with XPR1 variants that efficiently support X-MLV entry now provides the basis for studies on pathogenesis of these viruses, and such studies have now been initiated [123].

12. CAT-1 and XPR1 Receptor Downregulation by Env Glycoproteins in *Mus*

Virus entry can be inhibited by receptor mutants but can also be blocked by members of a second set of genes found in E-MLV- or X-MLV-infected wild mice. This family of resistance genes governs production of MLV Env glycoproteins that are thought to restrict virus through receptor interference. These genes include *Fv4*, which blocks E-MLVs [124], and the genes *Rmcf* and *Rmcf2* which restrict XP-MLVs and, in the case of *Rmcf*, inhibit P-MLV-induced disease [118, 124–126]. There is also evidence suggesting that additional *Rmcf*-like XPR1 receptor blocking genes are present in *M. castaneus* [127]. Specific ERVs have been mapped to 3 of these resistance genes, all of which are defective for virus production but have intact *env* genes. *Fv4* is a truncated provirus, *Rmcf* has a major deletion spanning *gag-pol* [124, 128], and *Rmcf2* has a stop codon that prematurely terminates integrase [125]. It has been proposed that the products of *Fv4*, *Rcmf*, and *Rcmf2* reduce or downregulate activity of their cognate receptors, and *Fv4* also has a defect in the fusion peptide of TM*env*, so

	C-term ECL3										ECL4										Virus infectivity				
	500										578										X-MLV	XMRV	Cz524	CasE # 1	P-MLV
Human	K	E	R	G	H	S	D	T	M	V	S	I	T	S	T	T	L	L	P	H	S	G	D	+++	+++	+++	+++	+++
Bat	.	.	Q	D	.	.	.	.	K	.	.	V	.	.	.	.	.	M	.	.	T	.	.	+++	+++	+++	+++	−
Guinea pig	.	.	L	.	.	.	.	.	I	.	.	.	.	T	M	P	T	.	.	.	.	.	.	+++	++	++	−	−
Dog	.	.	.	.	.	.	.	.	.	.	.	S	.	.	M	.	S	.	.	.	.	.	.	+++	++	−	−	−
Gerbil	.	.	.	.	.	.	.	Y	K	.	.	.	.	V	.	A	.	Q	.	.	V	.	.	+++	−	−	−	−
Ch. hamster	.	.	.	Q	.	.	.	.	.	.	.	.	.	A	.	A	F	Q	.	.	V	.	.	+	−	−	−	−
Syr. hamster	.	.	.	Q	.	.	.	.	.	.	.	.	.	V	.	.	F	Q	.	.	V	.	.	+	−	−	−	−

Figure 9: Susceptibility of various mammalian cells to XP-MLVs. Infectivity is measured as the $\log_{10}$ titer of FFU/100 μL of viral Env pseudotypes carrying the LacZ reporter. Log_{10} titer: +++, >3; ++, 2-3; +, 1-2. Infectivity of Chinese hamster cells can be increased by treatment with glycosylation inhibitors. Amino acid sequences are shown for the receptor determining regions of ECL3 and ECL4.

incorporation of this Env into virions in virus-infected cells results in their reduced infectivity [129].

Interference genes that target both host range types are found in the Asian species *M. castaneus*, mice that are infected with X-MLVs as well as with E-MLVs that are related to the leukemogenic and neuropathic CasBrE E-MLV [76, 80, 130]. These mice rely on several survival strategies to mitigate the consequences of infection. In addition to their restrictive *Xpr1*^{c} receptor, these mice carry *Fv4* as well as *Rmcf*-type XP-MLV interference genes. These interference genes likely arose in this species [80, 125]; CasBrE and *Fv4* have related Env genes (Figure 3) [130]. Transmission into other gene pools can proceed quickly for invasive genes like retrotransposons and for virus restriction genes that provide an immediate survival advantage. While the number and geographic distribution of *Rmcf*-type genes in Asian mice is not known, *Fv4* is found in AKV MLV-infected mice trapped in Japan (*M. molossinus)* and in Korea [80]. CasBrE and *Fv4* are both found in California mice, where Asian mice were likely introduced by the shipping trade [76, 82, 131]. The discovery of multiple interfering loci in infected mouse species and their geographic spread suggests that these coopted Env genes represent an effective survival mechanism. The general importance of this form of innate immunity is also illustrated by the fact that Env genes with similar antiviral functions have also been identified in chickens, sheep, and cats [132–134].

13. XPR1 Receptor Polymorphism and Entry Phenotypes in Non-*Mus* Species

The XP-MLVs are capable of infecting cells of other species, including humans (Figure 9). Cells of nearly all mammals are permissive to infection by X-MLVs, whereas a subset of these species is also susceptible to P-MLVs. This suggests that X-MLVs have less stringent receptor requirements than P-MLVs [17, 87, 88]. Some mammalian species show distinctive patterns of virus susceptibility not found in mice, for example, the restriction of P-MLVs and both wild mouse XP-MLVs by dog and buffalo cells (Figure 9) [17]. Analysis of mammalian XPR1 genes reveals significant sequence variability especially in the receptor determining ECL4, although this 13 residue segment contains 3 nonvariant residues, S578, T580, and G589. These conserved residues do not contribute to the receptor attachment site [17]. Further analysis of these functionally distinctive XPR1 genes may provide insight into the factors that facilitate transspecies transmission.

14. XMRV

Mice are important vectors of diseases that infect humans and their livestock [135], and MLV-infected house mouse species have a worldwide geographic distribution [136]. The horizontal transfer of infectious MLVs between individuals has been documented in wild mouse populations and in laboratory mice [82, 137], and MLV-related viral sequences, proteins, and antibodies have been reported in human blood donors and patients with prostate cancer and chronic fatigue syndrome [138–140]. An infectious virus first identified in prostate cancer patients, termed XMRV (xenotropic murine leukemia virus-related virus), shows close sequence homology with XP-MLVs [141], uses the XPR1 receptor [138], and has xenotropic host range [94]. Although XMRV origin by transspecies transmission is consistent with the evidence of MLV transmission between mice and evidence of transmission of mouse C-type viruses to other species [142–144], several recent studies on XMRV have implicated laboratory contamination [145–148]. Additional studies aiming to resolve the origins issue are focused on patient samples and the characterization of mice for XMRV-related sequences [149].

It is clear that XMRV differs from MLVs isolated from mice in several biological properties, including host range and receptor usage. The two critical residues for X-MLV entry in *Mus* XPR1, K500 and T582 [99], independently produce equivalent receptor determinants for X-MLV but not for XMRV [17] or for the wild mouse isolate CasE#1 [16]. While T582 but not K500 is required for CasE#1, XMRV preferentially relies on K500 [16, 17]. XMRV also differs from XP-MLVs in its ability to infect cells of different mammalian species. Although X-MLVs are able to infect all mammals, XMRV is uniquely restricted by cells of 3 species: Chinese hamster, Syrian hamster, and gerbil (Figure 9). For gerbil, this difference is likely attributable to XPR1 receptor polymorphism, as expression of the gerbil XPR1 receptor in heterologous cells reproduced the gerbil susceptibility pattern [17]. The restriction of XMRV in Syrian hamster BHK cells may involve other host factors. Expression of

human *Xpr1* in these cells resulted in susceptibility to X-MLV but not XMRV, and analysis of interspecies somatic cell hybrids suggested that BHK cells lack a secondary factor needed for XMRV infection [150]. These results suggest that XMRV differs from other X-MLVs in its interaction with XPR1 receptor determinants and also suggest that XMRV may be uniquely dependent on an as yet unidentified receptor cofactor. Further studies with this virus may provide additional insight into xenotropism and the interactions and identity of viral and host proteins that direct entry.

15. Conclusions

Retrovirus entry is dependent on the presence and accessibility of specific cell surface receptors. Mutational changes in these receptors and in the receptor attachment sites in the virus Env can alter the very first step in the virus life cycle and can thus have profound consequences for virus replication. Inhibition of virus entry has been a particularly effective antiviral tactic in mice infected with MLVs as well as with other gammaretroviruses [151]. Entry is also the target of host restrictions in other species subject to retrovirus infection as shown by the discovery of interfering ERV Envs in multiple species [132–134] and by the discovery of inhibitory mutations in other receptors, such as the HIV-1 CCR5 coreceptor [152].

For the laboratory mouse MLVs, alterations in host receptors and/or virus Env can result in virus restriction, can alter the type and tempo of infection-induced pathology, and may also influence postentry events [153]. The interacting sites of these receptors and Env are highly polymorphic, as expected for coevolving entities in an "arms race" driven by sequential reciprocal adaptations. For the XP-MLVs and XMRV, the battleground at the cell surface has produced 5 functionally distinct receptors in mice and more than a half dozen distinctive host range virus variants, variants that interact with different but overlapping sets of determinants on the XPR1 receptor or rely on alternative mechanisms of transmission independent of XPR1. For the E-MLVs, CAT-1 shows more limited variation although multiple viral Env subtypes have evolved. The interacting interfaces of virus and host proteins are targeted not just by mutational changes but also by epigenetic modifications resulting from glycosylation and by a host defensive strategy that relies on co-option of germline *env* genes to interfere with virus infection. There is also evidence of additional host factors or co-factors that influence entry of the mouse gammaretroviruses, some of which are affected by glycosylation [150, 154]. Future studies on the mouse gammaretroviruses should identify other host factors involved in entry and trans-species transmission, should describe the consequences of X-MLV infection in mice permissive to the "xenotropic" viruses, and should further illuminate the coevolutionary paths of these pathogens and their hosts.

Acknowledgments

This work was supported by the Intramural Research Program of the NIAID, NIH. The author would like to thank Reza Sadjapour, Carrie Martin, and Michael Dolan for assistance with figure preparation.

References

[1] C. Stocking and C. A. Kozak, "Murine endogenous retroviruses," *Cellular and Molecular Life Sciences*, vol. 65, no. 21, pp. 3383–3398, 2008.

[2] J. A. Levy and T. Pincus, "Demonstration of biological activity of a murine leukemia virus of New Zealand black mice," *Science*, vol. 170, no. 3955, pp. 326–327, 1970.

[3] P. J. Fischinger, S. Nomura, and D. P. Bolognesi, "A novel murine oncornavirus with dual eco- and xenotropic properties," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 72, no. 12, pp. 5150–5155, 1975.

[4] J. W. Hartley, N. K. Wolford, L. J. Old, and W. P. Rowe, "A new class of murine leukemia virus associated with development of spontaneous lymphomas," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 74, no. 2, pp. 789–792, 1977.

[5] C. A. Kozak, "Susceptibility of wild mouse cells to exogenous infection with xenotropic leukemia viruses: control by a single dominant locus on chromosome 1," *Journal of Virology*, vol. 55, no. 3, pp. 690–695, 1985.

[6] C. S. Tailor, A. Nouri, C. G. Lee, C. Kozak, and D. Kabat, "Cloning and characterization of a cell surface receptor for xenotropic and polytropic marine leukemia viruses," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 3, pp. 927–932, 1999.

[7] Y.-L. Yang, L. Guo, S. Xu et al., "Receptors for polytropic and xenotropic mouse leukaemia viruses encoded by a single gene at *Rmc1*," *Nature Genetics*, vol. 21, no. 2, pp. 216–219, 1999.

[8] J.-L. Battini, J. E. J. Rasko, and A. D. Miller, "A human cell-surface receptor for xenotropic and polytropic murine leukemia viruses: possible role in G protein-coupled signal transduction," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 4, pp. 1385–1390, 1999.

[9] J. L. Battini, O. Danos, and J. M. Heard, "Receptor-binding domain of murine leukemia virus envelope glycoproteins," *Journal of Virology*, vol. 69, no. 2, pp. 713–719, 1995.

[10] J. M. Heard and O. Danos, "An amino-terminal fragment of the Friend murine leukemia virus envelope glycoprotein binds the ecotropic receptor," *Journal of Virology*, vol. 65, no. 8, pp. 4026–4032, 1991.

[11] J. L. Battini, J. M. Heard, and O. Danos, "Receptor choice determinants in the envelope glycoproteins of amphotropic, xenotropic, and polytropic murine leukemia viruses," *Journal of Virology*, vol. 66, no. 3, pp. 1468–1475, 1992.

[12] J. A. Levy, "Xenotropic type C viruses," *Current Topics in Microbiology and Immunology*, vol. 79, pp. 111–213, 1978.

[13] M. V. Eiden, K. Farrell, J. Warsowe, L. C. Mahan, and C. A. Wilson, "Characterization of a naturally occurring ecotropic receptor that does not facilitate entry of all ecotropic murine retroviruses," *Journal of Virology*, vol. 67, no. 7, pp. 4056–4061, 1993.

[14] C. A. Kozak, "Genetic mapping of a mouse chromosomal locus required for mink cell focus-forming virus replication," *Journal of Virology*, vol. 48, no. 1, pp. 300–303, 1983.

[15] M. S. Lyu and C. A. Kozak, "Genetic basis for resistance to polytropic murine leukemia viruses in the wild mouse species *Mus castaneus*," *Journal of Virology*, vol. 70, no. 2, pp. 830–833, 1996.

[16] Y. Yan, R. C. Knoper, and C. A. Kozak, "Wild mouse variants of envelope genes of xenotropic/polytropic mouse gammaretroviruses and their XPR1 receptors elucidate receptor determinants of virus entry," *Journal of Virology*, vol. 81, no. 19, pp. 10550–10557, 2007.

[17] Y. Yan, Q. Liu, K. Wollenberg, C. Martin, A. Buckler-White, and C. A. Kozak, "Evolution of functional and sequence variants of the mammalian XPR1 receptor for mouse xenotropic gammaretroviruses and the human-derived retrovirus XMRV," *Journal of Virology*, vol. 84, no. 22, pp. 11970–11980, 2010.

[18] L. M. Albritton, L. Tseng, D. Scadden, and J. M. Cunningham, "A putative murine ecotropic retrovirus receptor gene encodes a multiple membrane-spanning protein and confers susceptibility to virus infection," *Cell*, vol. 57, no. 4, pp. 659–666, 1989.

[19] J. W. Kim, E. I. Closs, L. M. Albritton, and J. M. Cunningham, "Transport of cationic amino acids by the mouse ecotropic retrovirus receptor," *Nature*, vol. 352, no. 6337, pp. 725–728, 1991.

[20] H. Wang, M. P. Kavanaugh, R. A. North, and D. Kabat, "Cell-surface receptor for ecotropic murine retroviruses is a basic amino-acid transporter," *Nature*, vol. 352, no. 6337, pp. 729–731, 1991.

[21] J. Overbaugh, A. D. Miller, and M. V. Eiden, "Receptors and entry cofactors for retroviruses include single and multiple transmembrane-spanning proteins as well as newly described glycophosphatidylinositol-anchored and secreted proteins," *Microbiology and Molecular Biology Reviews*, vol. 65, no. 3, pp. 371–389, 2001.

[22] C. S. Tailor, D. Lavillette, M. Marin, and D. Kabat, "Cell surface receptors for gammaretroviruses," *Current Topics in Microbiology and Immunology*, vol. 281, pp. 29–106, 2003.

[23] T. A. Ericsson, Y. Takeuchi, C. Templin et al., "Identification of receptors for pig endogenous retrovirus," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 11, pp. 6759–6764, 2003.

[24] S. Hein, V. Prassolov, Y. Zhang et al., "Sodium-dependent myo-inositol transporter 1 is a cellular receptor for Mus cervicolor M813 murine leukemia virus," *Journal of Virology*, vol. 77, no. 10, pp. 5926–5932, 2003.

[25] R. Mendoza, M. M. Anderson, and J. Overbaugh, "A putative thiamine transport protein is a receptor for feline leukemia virus subgroup A," *Journal of Virology*, vol. 80, no. 7, pp. 3378–3385, 2006.

[26] A. D. Miller, U. Bergholz, M. Ziegler, and C. Stocking, "Identification of the myelin protein plasmolipin as the cell entry receptor for Mus caroli endogenous retrovirus," *Journal of Virology*, vol. 82, no. 14, pp. 6862–6868, 2008.

[27] L. M. Albritton, J. W. Kim, L. Tseng, and J. M. Cunningham, "Envelope-binding domain in the cationic amino acid transporter determines the host range of ecotropic murine retroviruses," *Journal of Virology*, vol. 67, no. 4, pp. 2091–2096, 1993.

[28] T. Yoshimoto, E. Yoshimoto, and D. Meruelo, "Identification of amino acid residues critical for infection with ecotropic murine leukemia retrovirus," *Journal of Virology*, vol. 67, no. 3, pp. 1310–1314, 1993.

[29] J. W. Kim and J. M. Cunningham, "N-linked glycosylation of the receptor for murine ecotropic retroviruses is altered in virus-infected cells," *Journal of Biological Chemistry*, vol. 268, no. 22, pp. 16316–16320, 1993.

[30] M. Pizzato, S. A. Marlow, E. D. Blair, and Y. Takeuchi, "Initial binding of murine leukemia virus particles to cells does not require specific Env-receptor interaction," *Journal of Virology*, vol. 73, no. 10, pp. 8599–8611, 1999.

[31] A. Jinno-Oue, M. Oue, and S. K. Ruscetti, "A unique heparin-binding domain in the envelope protein of the neuropathogenic PVC-211 murine leukemia virus may contribute to its brain capillary endothelial cell tropism," *Journal of Virology*, vol. 75, no. 24, pp. 12439–12445, 2001.

[32] P. Kumar, D. Nachagari, C. Fields, J. Franks, and L. M. Albritton, "Host cell cathepsins potentiate moloney murine leukemia virus infection," *Journal of Virology*, vol. 81, no. 19, pp. 10506–10514, 2007.

[33] B. H. Park, B. Matuschke, E. Lavi, and G. N. Gaulton, "A point mutation in the *env* gene of a murine leukemia virus induces syncytium formation and neurologic disease," *Journal of Virology*, vol. 68, no. 11, pp. 7516–7524, 1994.

[34] A. J. Mackrell, N. W. Soong, C. M. Curtis, and W. French Anderson, "Identification of a subdomain in the Moloney murine leukemia virus envelope protein involved in receptor binding," *Journal of Virology*, vol. 70, no. 3, pp. 1768–1774, 1996.

[35] R. A. Davey, Y. Zuo, and J. M. Cunningham, "Identification of a receptor-binding pocket on the envelope protein of Friend murine leukemia virus," *Journal of Virology*, vol. 73, no. 5, pp. 3758–3763, 1999.

[36] D. Fass, R. A. Davey, C. A. Hamson, P. S. Kim, J. M. Cunningham, and J. M. Berger, "Structure of a murine leukemia virus receptor-binding glycoprotein 2.0 angstrom resolution," *Science*, vol. 277, no. 5332, pp. 1662–1666, 1997.

[37] Y. Bae, S. M. Kingsman, and A. J. Kingsman, "Functional dissection of the Moloney murine leukemia virus envelope protein gp70," *Journal of Virology*, vol. 71, no. 3, pp. 2092–2099, 1997.

[38] D. Lavillette, A. Ruggieri, S. J. Russell, and F. L. Cosset, "Activation of a cell entry pathway common to type C mammalian retroviruses by soluble envelope fragments," *Journal of Virology*, vol. 74, no. 1, pp. 295–304, 2000.

[39] T. Zavorotinskaya and L. M. Albritton, "Suppression of a fusion defect by second site mutations in the ecotropic murine leukemia virus surface protein," *Journal of Virology*, vol. 73, no. 6, pp. 5034–5042, 1999.

[40] D. Lavillette, M. Maurice, C. Roche, S. J. Russell, M. Sitbon, and F. L. Cosset, "A proline-rich motif downstream of the receptor binding domain modulates conformation and fusogenicity of murine retroviral envelopes," *Journal of Virology*, vol. 72, no. 12, pp. 9955–9965, 1998.

[41] A. Pinter, R. Kopelman, Z. Li, S. C. Kayman, and D. A. Sanders, "Localization of the labile disulfide bond between SU and TM of the murine leukemia virus envelope protein complex to a highly conserved CWLC motif in SU that resembles the active-site sequence of thiol-disulfide exchange enzymes," *Journal of Virology*, vol. 71, no. 10, pp. 8073–8077, 1997.

[42] D. Lavillette, B. Boson, S. J. Russell, and F. L. Cosset, "Activation of membrane fusion by murine leukemia viruses is controlled in *cis* or in *trans* by interactions between the receptor-binding domain and a conserved disulfide loop of the carboxy terminus of the surface glycoprotein," *Journal of Virology*, vol. 75, no. 8, pp. 3685–3695, 2001.

[43] M. D. Burkhart, P. D'Agostino, S. C. Kayman, and A. Pinter, "Involvement of the C-terminal disulfide-bonded loop of murine leukemia virus SU protein in a postbinding step critical for viral entry," *Journal of Virology*, vol. 79, no. 12, pp. 7868–7876, 2005.

[44] Y. Yan and C. A. Kozak, "Novel postentry resistance to AKV ecotropic mouse gammaretroviruses in the African pygmy mouse, *Mus minutoides*," *Journal of Virology*, vol. 82, no. 13, pp. 6120–6129, 2008.

[45] Y. T. Jung and C. A. Kozak, "Generation of novel syncytium-inducing and host range variants of ecotropic Moloney murine leukemia virus in *Mus spicilegus*," *Journal of Virology*, vol. 77, no. 9, pp. 5065–5072, 2003.

[46] A. Ishimoto, "Infectivity of friend murine leukemia virus for hamster cells," *Journal of the National Cancer Institute*, vol. 74, no. 4, pp. 905–908, 1985.

[47] M. Masuda, C. A. Hanson, P. M. Hoffman et al., "Analysis of the unique hamster cell tropism of ecotropic murine leukemia virus PVC-211," *Journal of Virology*, vol. 70, no. 12, pp. 8534–8539, 1996.

[48] Y. T. Jung, T. Wu, and C. A. Kozak, "Novel host range and cytopathic variant of ecotropic Friend murine leukemia virus," *Journal of Virology*, vol. 78, no. 22, pp. 12189–12197, 2004.

[49] Y. Yan, Y. T. Jung, T. Wu, and C. A. Kozak, "Role of receptor polymorphism and glycosylation in syncytium induction and host range variation of ecotropic mouse gammaretroviruses," *Retrovirology*, vol. 5, article 2, 2008.

[50] S. K. Weller and H. M. Temin, "Cell killing by avian leukosis viruses," *Journal of Virology*, vol. 39, no. 3, pp. 713–721, 1981.

[51] J. L. Rohn, M. S. Moser, S. R. Gwynn, D. N. Baldwin, and J. Overbaugh, "In vivo evolution of a novel, syncytium-inducing and cytopathic feline leukemia virus variant," *Journal of Virology*, vol. 72, no. 4, pp. 2686–2696, 1998.

[52] C. Cheng-Mayer, D. Seto, M. Tateno, and J. A. Levy, "Biologic features of HIV-1 that correlate with virulence in the host," *Science*, vol. 240, no. 4848, pp. 80–82, 1988.

[53] H. M. Temin, "Mechanisms of cell killing/cytopathic effects by nonhuman retroviruses," *Reviews of Infectious Diseases*, vol. 10, no. 2, pp. 399–405, 1988.

[54] S. L. Murphy, M. Chung-Landers, M. Honczarenko, and G. N. Gaulton, "Linkage of reduced receptor affinity and superinfection to pathogenesis of TR1.3 murine leukemia virus," *Journal of Virology*, vol. 80, no. 9, pp. 4601–4609, 2006.

[55] L. Poliquin, D. Bergeron, J. L. Fortier, Y. Paquette, R. Bergeron, and E. Rassart, "Determinants of thymotropism in Kaplan radiation leukemia virus and nucleotide sequence of its envelope region," *Journal of Virology*, vol. 66, no. 8, pp. 5141–5146, 1992.

[56] S. L. Murphy, M. J. Honczarenko, N. V. Dugger, P. M. Hoffman, and G. N. Gaulton, "Disparate regions of envelope protein regulate syncytium formation versus spongiform encephalopathy in neurological disease induced by murine leukemia virus TR," *Journal of Virology*, vol. 78, no. 15, pp. 8392–8399, 2004.

[57] L. DesGroseillers, M. Barrette, and P. Jolicoeur, "Physical mapping of the paralysis-inducing determinant of a wild mouse ecotropic neurotropic retrovirus," *Journal of Virology*, vol. 52, no. 2, pp. 356–363, 1984.

[58] Y. Li, S. M. Cardona, R. S. Traister, and W. P. Lynch, "Retrovirus-induced spongiform neurodegeneration is mediated by unique central nervous system viral targeting and expression of Env alone," *Journal of Virology*, vol. 85, no. 5, pp. 2060–2078, 2011.

[59] M. Masuda, C. A. Hanson, W. G. Alvord, P. M. Hoffman, S. K. Ruscetti, and M. Masuda, "Effects of subtle changes in the SU protein of ecotropic murine leukemia virus on its brain capillary endothelial cell tropism and interference properties," *Virology*, vol. 215, no. 2, pp. 142–151, 1996.

[60] S. Olofsson and T. Bergström, "Glycoconjugate glycans as viral receptors," *Annals of Medicine*, vol. 37, no. 3, pp. 154–172, 2005.

[61] H. Wang, E. Klamo, S. E. Kuhmann, S. L. Kozak, M. P. Kavanaugh, and D. Kabat, "Modulation of ecotropic murine retroviruses by N-linked glycosylation of the cell surface receptor/amino acid transporter," *Journal of Virology*, vol. 70, no. 10, pp. 6884–6891, 1996.

[62] M. V. Eiden, K. Farrell, and C. A. Wilson, "Glycosylation-dependent inactivation of the ecotropic murine leukemia virus receptor," *Journal of Virology*, vol. 68, no. 2, pp. 626–631, 1994.

[63] C. A. Wilson and M. V. Eiden, "Viral and cellular factors governing hamster cell infection by murine and gibbon ape leukemia viruses," *Journal of Virology*, vol. 65, no. 11, pp. 5975–5982, 1991.

[64] D. G. Miller and A. D. Miller, "Tunicamycin treatment of CHO cells abrogates multiple blocks to retrovirus infection, one of which is due to a secreted inhibitor," *Journal of Virology*, vol. 66, no. 1, pp. 78–84, 1992.

[65] N. Tavoloni and A. Rudenholz, "Variable transduction efficiency of murine leukemia retroviral vector on mammalian cells: role of cellular glycosylation," *Virology*, vol. 229, no. 1, pp. 49–56, 1997.

[66] Y. Kubo, T. Ono, M. Ogura, A. Ishimoto, and H. Amanuma, "A glycosylation-defective variant of the ecotropic murine retrovirus receptor is expressed in rat XC cells," *Virology*, vol. 303, no. 2, pp. 338–344, 2002.

[67] Y. Kubo, A. Ishimoto, and H. Amanuma, "N-linked glycosylation is required for XC cell-specific syncytium formation by the peptide-containing envelope protein of ecotropic murine leukemia viruses," *Journal of Virology*, vol. 77, no. 13, pp. 7510–7516, 2003.

[68] R. A. Ogert, M. K. Lee, W. Ross, A. Buckler-White, M. A. Martin, and M. W. Cho, "N-linked glycosylation sites adjacent to and within the V1/V2 and the V3 loops of dualtropic human immunodeficiency virus type 1 isolate DH12 gp120 affect coreceptor usage and cellular tropism," *Journal of Virology*, vol. 75, no. 13, pp. 5998–6006, 2001.

[69] A. M. Schultz and S. Oroszlan, "Tunicamycin inhibits glycosylation of precursor polyprotein encoded by env gene of Rauscher murine leukemia virus," *Biochemical and Biophysical Research Communications*, vol. 86, no. 4, pp. 1206–1213, 1979.

[70] R. H. Felkner and M. J. Roth, "Mutational analysis of the N-linked glycosylation sites of the SU envelope protein of Moloney murine leukemia virus," *Journal of Virology*, vol. 66, no. 7, pp. 4258–4264, 1992.

[71] S. C. Kayman, R. Kopelman, S. Projan, D. M. Kinney, and A. Pinter, "Mutational analysis of N-linked glycosylation sites of Friend murine leukemia virus envelope protein," *Journal of Virology*, vol. 65, no. 10, pp. 5323–5332, 1991.

[72] Z. Li, A. Pinter, and S. C. Kayman, "The critical N-linked glycan of murine leukemia virus envelope protein promotes both folding of the C-terminal domains of the precursor polyprotein and stability of the postcleavage envelope complex," *Journal of Virology*, vol. 71, no. 9, pp. 7012–7019, 1997.

[73] K. B. Andersen, "A domain of murine retrovirus surface protein gp70 mediates cell fusion, as shown in a novel SC-1 cell fusion system," *Journal of Virology*, vol. 68, no. 5, pp. 3175–3182, 1994.

[74] J. L. Battini, S. C. Kayman, A. Pinter, J. M. Heard, and O. Danos, "Role of N-linked glycosylation in the activity of the

Friend murine leukemia virus SU protein receptor-binding domain," *Virology*, vol. 202, no. 1, pp. 496–499, 1994.

[75] R. C. Knoper, J. Ferrarone, Y. Yan, B. A. P. Lafont, and C. A. Kozak, "Removal of either N-glycan site from the envelope receptor binding domain of Moloney and Friend but not AKV mouse ecotropic gammaretroviruses alters receptor usage," *Virology*, vol. 391, no. 2, pp. 232–239, 2009.

[76] C. A. Kozak and R. R. O'Neill, "Diverse wild mouse origins of xenotropic, mink cell focus-forming, and two types of ecotropic proviral genes," *Journal of Virology*, vol. 61, no. 10, pp. 3082–3088, 1987.

[77] N. A. Jenkins, N. G. Copeland, B. A. Taylor, and B. K. Lee, "Organization, distribution, and stability of endogenous ecotropic murine leukemia virus DNA sequences in chromosomes of *Mus musculus*," *Journal of Virology*, vol. 43, no. 1, pp. 26–36, 1982.

[78] C. A. Kozak and W. P. Rowe, "Genetic mapping of ecotropic murine leukemia virus-inducing loci in six inbred strains," *Journal of Experimental Medicine*, vol. 155, no. 2, pp. 524–534, 1982.

[79] C. A. Kozak and S. Ruscetti, "Retroviruses in rodents," in *The Retroviridae*, J. Levy, Ed., vol. III, Plenum Press, New York, NY, USA, 1994.

[80] Y. Inaguma, N. Miyashita, K. Moriwaki et al., "Acquisition of two endogenous ecotropic murine leukemia viruses in distinct Asian wild mouse populations," *Journal of Virology*, vol. 65, no. 4, pp. 1796–1802, 1991.

[81] M. B. Gardner, V. Klement, and R. R. Rongey, "Type C virus expression in lymphoma paralysis prone wild mice," *Journal of the National Cancer Institute*, vol. 57, no. 3, pp. 585–590, 1976.

[82] M. B. Gardner, A. Chiri, and M. F. Dougherty, "Congenital transmission of murine leukemia virus from wild mice prone to the development of lymphoma and paralysis," *Journal of the National Cancer Institute*, vol. 62, no. 1, pp. 63–70, 1979.

[83] E. Rassart, L. Nelbach, and P. Jolicoeur, "Cas-Br-E murine leukemia virus: sequencing of the paralytogenic regions of its genome and derivation of specific probes to study its origin and the structure of its recombinant genomes in leukemic tissues," *Journal of Virology*, vol. 60, no. 3, pp. 910–919, 1986.

[84] A. Orth, T. Adama, W. Din, F. Bonhomme, and R. S. Singh, "Natural hybridation between two domestic mouse subspecies, *Mus musculus domesticus* and *Mus musculus castaneus* near by Lake Casitas, California," *Genome*, vol. 41, no. 1, pp. 104–110, 1998.

[85] P. Voytek and C. A. Kozak, "Nucleotide sequence and a mode of transmission of the wild mouse ecotropic virus, HoMuLV," *Virology*, vol. 173, no. 1, pp. 58–67, 1989.

[86] M. R. Lander and S. K. Chattopadhyay, "A *mus dunni* cell line that lacks sequences closely related to endogenous murine leukemia viruses and can be infected by ecotropic, amphotropic, xenotropic, and mink cell focus-forming viruses," *Journal of Virology*, vol. 52, no. 2, pp. 695–698, 1984.

[87] J. A. Levy, "Host range of murine xenotropic virus: replication in avian cells," *Nature*, vol. 253, no. 5487, pp. 140–142, 1975.

[88] H. K. Oie, E. K. Russell, and J. H. Dotson, "Host range properties of murine xenotropic and ecotropic type C viruses," *Journal of the National Cancer Institute*, vol. 56, no. 2, pp. 423–426, 1976.

[89] M. W. Cloyd, M. M. Thompson, and J. W. Hartley, "Host range of mink cell focus-inducing viruses," *Virology*, vol. 140, no. 2, pp. 239–248, 1985.

[90] M. Vogt, C. Haggblom, S. Swift, and M. Haas, "Envelope gene and long terminal repeat determine the different biological properties of Rauscher, Friend, and Moloney mink cell focus-inducing viruses," *Journal of Virology*, vol. 55, no. 1, pp. 184–192, 1985.

[91] P. A. Chatis, C. A. Holland, and J. E. Silver, "A 3' end fragment encompassing the transcriptional enhancers of nondefective Friend virus confers erythroleukemogenicity on Moloney leukemia virus," *Journal of Virology*, vol. 52, no. 1, pp. 248–254, 1984.

[92] A. Ishimoto, A. Adachi, K. Sakai, and M. Matsuyama, "Long terminal repeat of friend-MCF virus contains the sequence responsible for erythroid leukemia," *Virology*, vol. 141, no. 1, pp. 30–42, 1985.

[93] S. Bahrami, M. Duch, and F. S. Pedersen, "Change of tropism of SL3-2 murine leukemia virus, using random mutational libraries," *Journal of Virology*, vol. 78, no. 17, pp. 9343–9351, 2004.

[94] Y. Yan, Q. Liu, and C. A. Kozak, "Six host range variants of the xenotropic/polytropic gammaretroviruses define determinants for entry in the XPR1 cell surface receptor," *Retrovirology*, vol. 6, p. 87, 2009.

[95] S. Baliji, Q. Liu, and C. A. Kozak, "Common inbred strains of the laboratory mouse that are susceptible to infection by mouse xenotropic gammaretroviruses and the human-derived retrovirus XMRV," *Journal of Virology*, vol. 84, no. 24, pp. 12841–12849, 2010.

[96] J. W. Hartley and W. P. Rowe, "Clonal cell lines from a feral mouse embryo which lack host range restrictions for murine leukemia viruses," *Virology*, vol. 65, no. 1, pp. 128–134, 1975.

[97] B. Chesebro and K. Wehrly, "Different murine cells lines manifest unique patterns of interference to superinfection by murine leukemia viruses," *Virology*, vol. 141, no. 1, pp. 119–129, 1985.

[98] P. Sharma, S. Patntirapong, S. Hann, and P. V. Hauschka, "RANKL-RANK signaling regulates expression of xenotropic and polytropic virus receptor (XPR1) in osteoclasts," *Biochemical and Biophysical Research Communications*, vol. 399, no. 2, pp. 129–132, 2010.

[99] M. Marin, C. S. Tailor, A. Nouri, S. L. Kozak, and D. Kabat, "Polymorphisms of the cell surface receptor control mouse susceptibilities to xenotropic and polytropic leukemia viruses," *Journal of Virology*, vol. 73, no. 11, pp. 9362–9368, 1999.

[100] H. C. I. Morse, "Introduction," in *Origins of Inbred Mice*, H. C. Morse, Ed., vol. III, pp. 1–31, Academic Press, New York, NY, USA, 1978.

[101] H. Yang, T. A. Bell, G. A. Churchill, and F. Pardo-Manuel De Villena, "On the subspecific origin of the laboratory mouse," *Nature Genetics*, vol. 39, no. 9, pp. 1100–1107, 2007.

[102] N. S. Van Hoeven and A. D. Miller, "Use of different but overlapping determinants in a retrovirus receptor accounts for non-reciprocal interference between xenotropic and polytropic murine leukemia viruses," *Retrovirology*, vol. 2, article 76, 2005.

[103] J. K. Brown, C. Fung, and C. S. Tailor, "Comprehensive mapping of receptor-functioning domains in feline leukemia virus subgroup C receptor FLVCR1," *Journal of Virology*, vol. 80, no. 4, pp. 1742–1751, 2006.

[104] K. B. Farrell, J. L. Russ, R. K. Murthy, and M. V. Eiden, "Reassessing the role of region A in Pit1-mediated viral entry," *Journal of Virology*, vol. 76, no. 15, pp. 7683–7693, 2002.

[105] N. Manel, J. L. Battini, and M. Sitbon, "Human T cell leukemia virus envelope binding and virus entry are mediated by distinct domains of the glucose transporter GLUT1," *Journal of Biological Chemistry*, vol. 280, no. 32, pp. 29025–29029, 2005.

[106] A. L. Barnett, R. A. Davey, and J. M. Cunningham, "Modular organization of the Friend murine leukemia virus envelope protein underlies the mechanism of infection," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 7, pp. 4113–4118, 2001.

[107] A. L. Barnett and J. M. Cunningham, "Receptor binding transforms the surface subunit of the mammalian C-type retrovirus envelope protein from an inhibitor to an activator of fusion," *Journal of Virology*, vol. 75, no. 19, pp. 9096–9105, 2001.

[108] D. L. Wensel, W. Li, and J. M. Cunningham, "A virus-virus interaction circumvents the virus receptor requirement for infection by pathogenic retroviruses," *Journal of Virology*, vol. 77, no. 6, pp. 3460–3469, 2003.

[109] W. Herr and W. Gilbert, "Free and integrated recombinant murine leukemia virus DNAs appear in preleukemic thymuses of AKR/J mice," *Journal of Virology*, vol. 50, no. 1, pp. 155–162, 1984.

[110] F. K. Yoshimura, T. Wang, F. Yu, H. R. C. Kim, and J. R. Turner, "Mink cell focus-forming murine leukemia virus infection induces apoptosis of thymic lymphocytes," *Journal of Virology*, vol. 74, no. 17, pp. 8119–8126, 2000.

[111] S. Nanua and F. K. Yoshimura, "Mink epithelial cell killing by pathogenic murine leukemia viruses involves endoplasmic reticulum stress," *Journal of Virology*, vol. 78, no. 21, pp. 12071–12074, 2004.

[112] J. P. Stoye, C. Moroni, and J. M. Coffin, "Virological events leading to spontaneous AKR thymomas," *Journal of Virology*, vol. 65, no. 3, pp. 1273–1285, 1991.

[113] P. Jern, J. P. Stoye, and J. M. Coffin, "Role of APOBEC3 in genetic diversity among endogenous murine leukemia viruses," *PLoS Genetics*, vol. 3, no. 10, article e183, 2007.

[114] P. J. Fischinger, C. S. Blevins, and N. M. Dunlop, "Genomic masking of nondefective recombinant murine leukemia virus in Moloney virus stocks," *Science*, vol. 201, no. 4354, pp. 457–459, 1978.

[115] M. Lavignon and L. Evans, "A multistep process of leukemogenesis in moloney murine leukemia virus-infected mice that is modulated by retroviral pseudotyping and interference," *Journal of Virology*, vol. 70, no. 6, pp. 3852–3862, 1996.

[116] L. H. Evans, A. S. Alamgir, N. Owens et al., "Mobilization of endogenous retroviruses in mice after infection with an exogenous retrovirus," *Journal of Virology*, vol. 83, no. 6, pp. 2429–2435, 2009.

[117] M. M. Anderson, A. S. Lauring, C. C. Burns et al., "Identification of a cellular cofactor required for infection by feline leukemia virus," *Science*, vol. 287, no. 5459, pp. 1828–1830, 2000.

[118] S. Ruscetti, L. Davis, J. Feild, and A. Oliff, "Friend murine leukemia virus-induced leukemia is associated with the formation of mink cell focus-inducing viruses and is blocked in mice expressing endogenous mink cell focus-inducing xenotropic viral envelope genes," *Journal of Experimental Medicine*, vol. 154, no. 3, pp. 907–920, 1981.

[119] P. Boursot, W. Din, R. Anand et al., "Origin and radiation of the house mouse: mitochondrial DNA phylogeny," *Journal of Evolutionary Biology*, vol. 9, no. 4, pp. 391–415, 1996.

[120] W. Din, R. Anand, P. Boursot et al., "Origin and radiation of the house mouse: clues from nuclear genes," *Journal of Evolutionary Biology*, vol. 9, no. 5, pp. 519–539, 1996.

[121] R. R. O'Neill, A. S. Khan, M. D. Hoggan et al., "Specific hybridization probes demonstrate fewer xenotropic than mink cell focus-forming murine leukemia virus *env*-related sequences in DNAs from inbred laboratory mice," *Journal of Virology*, vol. 58, no. 2, pp. 359–366, 1986.

[122] W. N. Frankel, J. P. Stoye, B. A. Taylor, and J. M. Coffin, "A linkage map of endogenous murine leukemia proviruses," *Genetics*, vol. 124, no. 2, pp. 221–236, 1990.

[123] T. Sakuma, J. M. Tonne, K. A. Squillace et al., "Early events in retrovirus XMRV infection of the wild-derived mouse *Mus pahari*," *Journal of Virology*, vol. 85, no. 3, pp. 1205–1213, 2011.

[124] H. Ikeda, F. Laigret, M. A. Martin, and R. Repaske, "Characterization of a molecularly cloned retroviral sequence associated with *Fv-4* resistance," *Journal of Virology*, vol. 55, no. 3, pp. 768–777, 1985.

[125] T. Wu, Y. Yan, and C. A. Kozak, "*Rmcf2*, a xenotropic provirus in the Asian mouse species *Mus castaneus*, blocks infection by polytropic mouse gammaretroviruses," *Journal of Virology*, vol. 79, no. 15, pp. 9677–9684, 2005.

[126] R. H. Bassin, S. Ruscetti, I. Ali, D. K. Haapala, and A. Rein, "Normal DBA/2 mouse cells synthesize a glycoprotein which interferes with MCF virus infection," *Virology*, vol. 123, no. 1, pp. 139–151, 1982.

[127] C. A. Kozak, "The mouse "xenotropic" gammaretroviruses and their XPR1 receptor," *Retrovirology*, vol. 7, article 101, 2010.

[128] Y. T. Jung, M. S. Lyu, A. Buckler-White, and C. A. Kozak, "Characterization of a polytropic murine leukemia virus proviral sequence associated with the virus resistance gene *Rmcf* of *DBA/2 mice*," *Journal of Virology*, vol. 76, no. 16, pp. 8218–8224, 2002.

[129] G. M. Taylor, Y. Gao, and D. A. Sanders, "Fv-4: identification of the defect in Env and the mechanism of resistance to ecotropic murine leukemia virus," *Journal of Virology*, vol. 75, no. 22, pp. 11244–11248, 2001.

[130] H. Ikeda, K. Kato, H. Kitani et al., "Virological properties and nucleotide sequences of cas-E-type endogenous ecotropic murine leukemia viruses in south asian wild mice, *Mus musculus castaneus*," *Journal of Virology*, vol. 75, no. 11, pp. 5049–5058, 2001.

[131] M. B. Gardner, S. Rasheed, and B. K. Pal, "Akvr-1, a dominant murine leukemia virus restriction gene, is polymorphic in leukemia-prone wild mice," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 77, no. 1, pp. 531–535, 1980.

[132] H. L. Robinson, S. M. Astrin, A. M. Senior, and F. H. Salazar, "Host susceptibility to endogenous viruses: defective, glycoprotein-expressing proviruses interfere with infections," *Journal of Virology*, vol. 40, no. 3, pp. 745–751, 1981.

[133] T. E. Spencer, M. Mura, C. A. Gray, P. J. Griebel, and M. Palmarini, "Receptor usage and fetal expression of ovine endogenous betaretroviruses: implications for coevolution of endogenous and exogenous retroviruses," *Journal of Virology*, vol. 77, no. 1, pp. 749–753, 2003.

[134] A. S. McDougall, A. Terry, T. Tzavaras, C. Cheney, J. Rojko, and J. C. Neil, "Defective endogenous proviruses are expressed in feline lymphoid cells: Evidence for a role in natural resistance to subgroup B feline leukemia viruses," *Journal of Virology*, vol. 68, no. 4, pp. 2151–2160, 1994.

[135] W. J. Weber, *Diseases Transmitted by Rats and Mice*, Thomas Publishers, Fresno, Calif, USA, 1982.

[136] J. Marshall, "Taxonomy," in *The Mouse in Biomedical Research*, S. J. H. L. Foster and J. G. Fox, Eds., vol. 1, pp. 17–26, Academic Press, New York, NY, USA, 1981.

[137] J. L. Portis, F. J. McAtee, and S. F. Hayes, "Horizontal transmission of murine retroviruses," *Journal of Virology*, vol. 61, no. 4, pp. 1037–1044, 1987.

[138] B. Dong, S. Kim, S. Hong et al., "An infectious retrovirus susceptible to an IFN antiviral pathway from human prostate tumors," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 5, pp. 1655–1660, 2007.

[139] V. C. Lombardi, F. W. Ruscetti, J. das Gupta et al., "Detection of an infectious retrovirus, XMRV, in blood cells of patients with chronic fatigue syndrome," *Science*, vol. 326, no. 5952, pp. 585–589, 2009.

[140] E. C. Knouf, M. J. Metzger, P. S. Mitchell et al., "Multiple integrated copies and high-level production of the human retrovirus XMRV (Xenotropic Murine leukemia virus-Related Virus) from 22Rv1 prostate carcinoma cells," *Journal of Virology*, vol. 83, no. 14, pp. 7353–7356, 2009.

[141] A. Urisman, R. J. Molinaro, N. Fischer et al., "Identification of a novel gammaretrovirus in prostate tumors of patients homozygous for R462Q RNASEL variant," *PLoS Pathogens*, vol. 2, no. 3, article e25, 2006.

[142] R. E. Benveniste and G. J. Todaro, "Homology between type C viruses of various species as determined by molecular hybridization," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 70, no. 12, pp. 3316–3320, 1973.

[143] R. E. Benveniste, R. Heinemann, and G. L. Wilson, "Detection of baboon type C viral sequences in various primate tissues by molecular hybridization," *Journal of Virology*, vol. 14, no. 1, pp. 56–67, 1974.

[144] M. M. Lieber, C. J. Sherr, and G. J. Todaro, "Isolation from the Asian mouse *Mus caroli* of an endogenous type C virus related to infectious primate type C viruses," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 72, no. 6, pp. 2315–2319, 1975.

[145] S. Hue, E. R. Gray, A. Gall et al., "Disease-associated XMRV sequences are consistent with laboratory contamination," *Retrovirology*, vol. 7, no. 1, article 111, 2010.

[146] M. J. Robinson, O. W. Erlwein, S. Kaye et al., "Mouse DNA contamination in human tissue tested for XMRV," *Retrovirology*, vol. 7, article 108, 2010.

[147] B. Oakes, A. K. Tai, O. Cingoz et al., "Contamination of human DNA samples with mouse DNA can lead to false detection of XMRV-like sequences," *Retrovirology*, vol. 7, article 109, 2010.

[148] E. Sato, R. A. Furuta, and T. Miyazawa, "An endogenous murine leukemia viral genome contaminant in a commercial RT-PCR Kit is amplified using standard primers for XMRV," *Retrovirology*, vol. 7, article 110, 2010.

[149] T. Paprotka, K. A. Delviks-Frankenberry, O. Cingöz et al., "Recombinant origin of the retrovirus XMRV," *Science*, vol. 333, no. 6038, pp. 97–101, 2011.

[150] W. Q. Xu and M. V. Eiden, "Primate gammaretroviruses require an ancillary factor not required for murine gammaretroviruses to infect BHK cells," *Journal of Virology*, vol. 85, no. 7, pp. 3498–3506, 2011.

[151] C. A. Wilson, K. B. Farrell, and M. V. Eiden, "Comparison of cDNAs encoding the gibbon ape leukaemia virus receptor from susceptible and non-susceptible murine cells," *Journal of General Virology*, vol. 75, no. 8, pp. 1901–1908, 1994.

[152] R. Liu, W. A. Paxton, S. Choe et al., "Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection," *Cell*, vol. 86, no. 3, pp. 367–377, 1996.

[153] N. M. M. Oliveira, R. Trikha, and Á. McKnight, "A novel envelope mediated post entry restriction of murine leukaemia virus in human cells is Ref1/TRIM5α independent," *Retrovirology*, vol. 7, article 81, 2010.

[154] D. G. Miller and A. D. Miller, "Inhibitors of retrovirus infection are secreted by several hamster cell lines and are also present in hamster sera," *Journal of Virology*, vol. 67, no. 9, pp. 5346–5352, 1993.

Hindawi Publishing Corporation
Advances in Virology
Volume 2011, Article ID 341294, 20 pages
doi:10.1155/2011/341294

Review Article

Phylogeny-Directed Search for Murine Leukemia Virus-Like Retroviruses in Vertebrate Genomes and in Patients Suffering from Myalgic Encephalomyelitis/Chronic Fatigue Syndrome and Prostate Cancer

Jonas Blomberg,[1,2] Ali Sheikholvaezin,[1] Amal Elfaitouri,[1] Fredrik Blomberg,[1] Anna Sjösten,[1] Johan Mattson Ulfstedt,[1] Rüdiger Pipkorn,[3] Clas Källander,[4] Christina Öhrmalm,[1] and Göran Sperber[5]

[1] *Section of Clinical Microbiology, Department of Medical Sciences, Uppsala University, 751 05 Uppsala, Sweden*
[2] *Clinical Microbiology, Academic Hospital, 751 85 Uppsala, Sweden*
[3] *Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 280, 69120 Heidelberg, Germany*
[4] *Cavidi Tech AB, Uppsala Science Park, 751 83 Uppsala, Sweden*
[5] *Department of Neuroscience, Biomedical Centre, Uppsala University, P.O. Box 593, 751 24 Uppsala, Sweden*

Correspondence should be addressed to Jonas Blomberg, jonas.blomberg@medsci.uu.se

Received 17 April 2011; Revised 11 June 2011; Accepted 22 June 2011

Academic Editor: Yoshinao Kubo

Gammaretrovirus-like sequences occur in most vertebrate genomes. Murine Leukemia Virus (MLV) like retroviruses (MLLVs) are a subset, which may be pathogenic and spread cross-species. Retroviruses highly similar to MLLVs (xenotropic murine retrovirus related virus (XMRV) and Human Mouse retrovirus-like RetroViruses (HMRVs)) reported from patients suffering from prostate cancer (PC) and myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) raise the possibility that also humans have been infected. Structurally intact, potentially infectious MLLVs occur in the genomes of some mammals, especially mouse. Mouse MLLVs contain three major groups. One, MERV G3, contained MLVs and XMRV/HMRV. Its presence in mouse DNA, and the abundance of xenotropic MLVs in biologicals, is a source of false positivity. Theoretically, XMRV/HMRV could be one of several MLLV transspecies infections. MLLV pathobiology and diversity indicate optimal strategies for investigating XMRV/HMRV in humans and raise ethical concerns. The alternatives that XMRV/HMRV may give a hard-to-detect "stealth" infection, or that XMRV/HMRV never reached humans, have to be considered.

1. Introduction

Recent reports of human gammaretroviruses highly similar to murine gammaretroviruses in PC and ME/CFS patients raise questions regarding (i) the occurrence of such retroviral sequences in murine and other vertebrate genomes, (ii) probable routes of spread of such viruses, and (iii) available methods for the detection of infection with them. In this review, we apply a phylogenetic aspect to the occurrence of XMRV/HMRV in genomes, and to the diagnostic search for it in humans. The comparative approach [1] can also enhance the study of pathobiology and epidemiology of XMRV/HMRV. Given the recent great activity in the field, the review cannot be exhaustive. Indeed, reports indicating that all XMRV/HMRV findings in humans may be due to different forms of laboratory contamination [2, 3] stress the need for a critical evaluation.

2. The Genus Gammaretrovirus

2.1. General. Murine leukemia viruses (MLVs) are gammaretroviruses which may be both exogenous (transmits between individuals, i.e., horizontally) or endogenous (proviruses integrated into the germ line of mice and

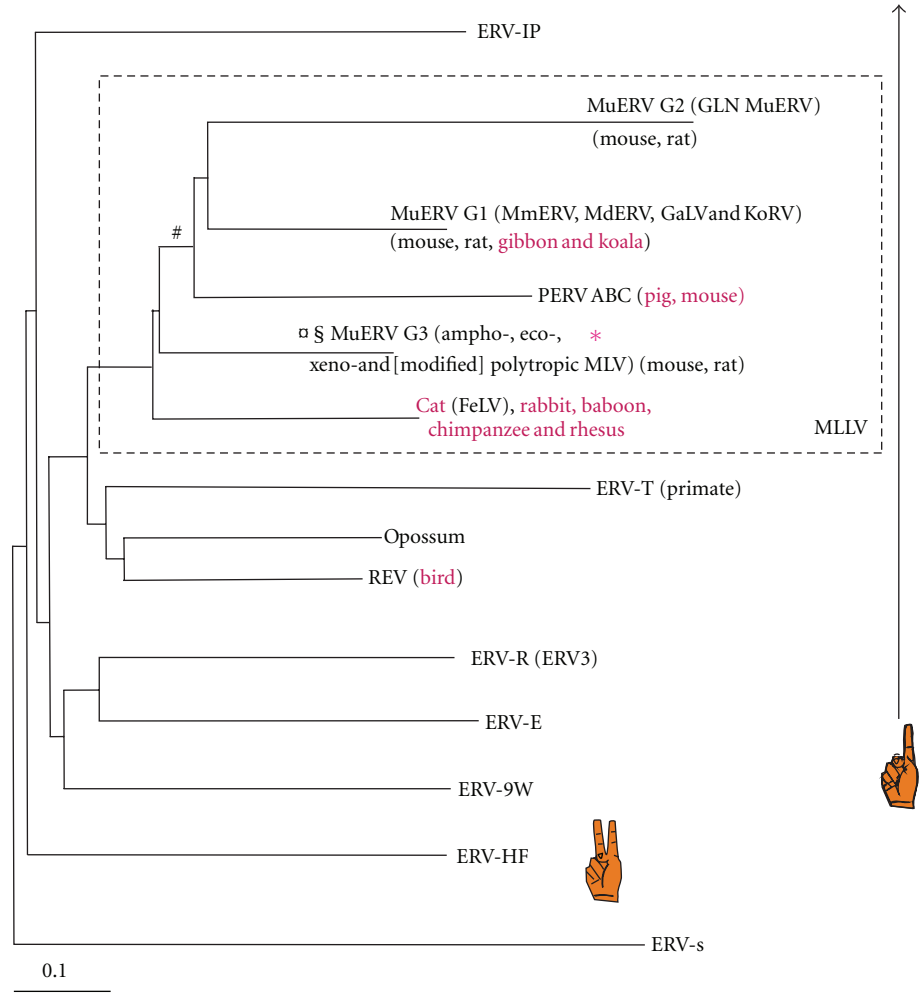


Figure 1: Simplified phylogeny of gammaretroviruses based on over 2000 gammaretrovirus-like sequences with one zinc finger in Gag, from selected vertebrate genomes (in a first version of RetroBank) and reference gammaretroviruses. Retroviral groups which occur in phylogenetically distant vertebrate hosts, indicating cross-species transmission events, are shown in red. The neighbor-joining tree was based on an alignment of 105 reference and consensus Pol amino acid sequences. Only the major branches are shown. As explained in the text, they were provisionally named after the cosegregating HERV group. The group of "MLV-like viruses," MLLVs, at least 60% similar in Pol, is boxed in. The similarity is calculated from the inverted ratio of the BLASTP score of the sequence against itself to another sequence, or to a consensus Pol sequence. Some two-zinc finger sequences (ERV-HF, containing HERV-H and HERV-F) were included as a reference. Although the clustering included several genomes, the groups were named from the human sequence representative. MuERV: mouse endogenous retrovirus. MmERV is from Bromham et al. [13], and MdERV is *Mus dunni* ERV from Wolgamot et al. [14]. GLN MuERV is from Ribet et al. [15]. Symbols: ∗ position of XMRV/HMRV, § recombinant MuERV Sp496-5Sb [16] from *Mus spretus, hortulanus* endogenous murine virus, HEMV, from *Mus spicilegus* [17], # MuRRS [18], and MuERVC [19]. An ERV-S sequence [20] was used to root the tree. It came from HERV-S, a sequence intermediate to spuma-, epsilon-, and gammaretroviruses. It belongs to the so-called "ERV3 family" according to the RepBase nomenclature [21]. This large clade is also called "Class III ERVs." It should not be confused with the ERV3 group shown in the figure. The hand symbols denote proviruses with one and two zinc fingers in Gag, respectively. The murine gammaretroviral groups G1-G3 are described in greater detail in a forthcoming paper (Elfaitouri et al., accepted, Plos One).

thereby being transmitted to the next generation, i.e., vertically). Gammaretroviruses were defined from exogenous retroviruses with MLV as a reference. The vast amount of genomic information now available shows that they are included in the large Class I ERV clade. Figure 1 depicts the major gammaretroviral groups, based on a clustering analysis of polymerase sequences of a large number of endogenous sequences. Using MLVs (Genbank ID J02255, Locus MLMCG) as a taxonomical starting point, gammaretroviruses related to MLVs can be labeled "MLV-like retroviruses" and are here referred to as "MLLVs." Endogenous versions of MLLVs occur in the genomes of some mammals and marsupials. The major branches segregated with previously characterized HERV groups, named after primer binding site, PBS, usage. This usage was found to be retained in the larger context (data not shown). These branches were therefore provisionally named after the respective HERV group. MLLVs are boxed in. They were

defined as a cluster of polymerase (Pol) sequences which were at least 60% similar, including the MLVs. The similarity was based on the BLASTP score of their Pol amino acid sequences to each other. The concept "MLLV" is justified for the purpose of this review to encompass similar proviruses of phylogenetically distant vertebrates. Their wide distribution, in pigs, primates, rodents, and koalas, demonstrates a tendency to interspecies spread, potentially relevant both for human disease [4–9] and xenotransplantation [10–12].

The human genome also contains remnants of infections with retroviruses highly related to MLLVs, HERV-T. However, these were integrated in the rather distant past. In contrast, MLLVs have repeatedly infected nonmurine vertebrates in the not so distant past, judging from the low degree of divergence of entire proviral and LTR sequences. Mediterranean and middle Eastern cats [22], turkeys [23], gibbon apes [24, 25], and koalas [26, 27] have been "invaded" by MLLVs. This is further discussed below. In some hosts, they are both endogenous and exogenous, in others just exogenous. In the infected animals, exogenous MLLVs are associated with significant disease like encephalitis, malignancy (leukemia and lymphoma), wasting, immunosuppression, and autoimmunity. This makes it especially important to establish if also the human species is now "invaded" by a murine MLLV, that is, XMRV/HMRV.

2.2. Properties

2.2.1. The Gammaretrovirus Genome. Gammaretroviruses have a simple genome (Figure 2), that is, there are no known additional overlapping reading frames for nonstructural regulatory proteins such as those which occur in betaretroviruses, deltaretroviruses, and lentiviruses. Moloney MLV is the reference gammaretrovirus [28]. Despite being simple, MLVs have some distinguishing features. The *gag* gene may have alternative translational start sites, giving rise to both a myristoylated Gag polyprotein, which contains the inner structural proteins, and a glycosylated Gag membrane protein. Many of them have a phosphoprotein following the matrix protein p15, called p12. The major Gag protein is p30, the capsid protein. It is responsible for many of the antigenic cross-reactions which gave rise to the acronym Gag ("group-specific antigen"). Further, like many other gammaretroviruses, the MLLVs have one zinc finger in the NucleoCapsid, NC, portion of Gag (the p10 protein), instead of the customary two [29]. The first finger is replaced with a highly charged sequence, binding to retroviral genomic RNA in a somewhat different way compared to two zinc finger retroviruses. The zinc finger status is here used as a taxonomic marker of a subset of gammaretroviruses [30]. All MLLVs have one zinc finger. Occasional readthrough of a Gag stop codon creates Gag-Pol polyproteins.

Another structural genomic aspect is that retroviruses with simple genomes like alpharetroviruses and gammaretroviruses occasionally may take up an oncogene in their genome, to form acutely transforming ("sarcoma") viruses [28]. They are often replication deficient. They then need a replication competent virus, a helper virus, to replicate. The so-called murine AIDS (MAIDS) virus variants are also defective, producing a new Gag protein (p60) which contains part of the p12 protein and a T cell neoepitope [31–33]. Likewise, the feline leukemiavirus immunodeficiency-inducing defective virus (FeLV-T) has a mutated Env [34]. Immunodeficiency associated with this variant is sometimes called "Feline AIDS," FAIDS, although the feline immunodeficiency virus can cause another form of (FAIDS). Recombination between exo- and endogenous MLLV sequences is common in both mice and cats [35].

MLVs can cause cancer in at least two ways, either through incorporation of an oncogene, or by integration near 5′ends of transcription units and associated CpG-rich portions [28]. The propensity to integrate into or next to promoters is a gammaretroviral specialty [36–38]. Random integration next to an oncogene is a frequent cause of leukemia in MLV-infected animals. Humans are not immune to this mechanism. MLV-based gene therapeutic vectors have the same target specificity [37, 39], see also [40]. Thus, an MLLV infecting humans would be expected to cause leukemias or lymphomas.

Finally, the envelope proteins (Surface Unit; SU, gp70 and TransMembrane protein; TM, p15E) are central for tissue tropism, immunogenicity, and for immunosuppression. The latter contains the conserved so-called "immunosuppressive domain" (ISD) [41–45] whose mode of action is still poorly known. Thus, despite their basic structural simplicity, MLLVs can display a complex pathobiology.

2.2.2. Occurrence among Vertebrates. A rich source of vertebrate information is the collection of ERV sequences in an early version of RetroBank [47]. The program RetroTector (ReTe) [48] was used to collect more than 40.000 proviruses from whole genome analyses of thirty vertebrate genomes. ReTe is based on a pattern recognition algorithm. It uses the order of and distances between conserved retroviral motifs to detect and characterize retroviral sequences from large genomic data sets. A score is calculated from the degree of fit to a collection of conserved motifs from all seven retroviral genera. The higher the score, the better the fit to a structural model which encompasses most orthoretroviral and also some retrovirus-like sequences. A provisional genus is designated by counting the best-fitting motifs from each genus.

Gammaretrovirus-like sequences were detected in all of the 30 genomes (those reported in [48] plus the turkey genome). Those scoring above 1000 by RetroTector, and with only one zinc finger (n = 2534, from marmoset 32, dog 41, guinea pig 211, horse 4, duckbill 16, lemur 43, orangutan 82, rhesus 204, pig 79, tree shrew 11, lizard 162, cow 37, human 143, opossum 393, mouse 515, chimpanzee 192, rat 361, and zebra finch 8), were selected from RetroBank. The mouse genome assembly employed was mm8, from a C57 black mouse. Some were from the MLLV subset, as defined in Figure 1. A study of their taxonomy was initiated by clustering and consensus sequence calculation at the 85% similarity level. It resulted in 75 interhost Pol consensus sequences which together with reference Pols were used to build the tree shown in the simplified form in Figure 1. This is part of JBs ongoing work with retroviral

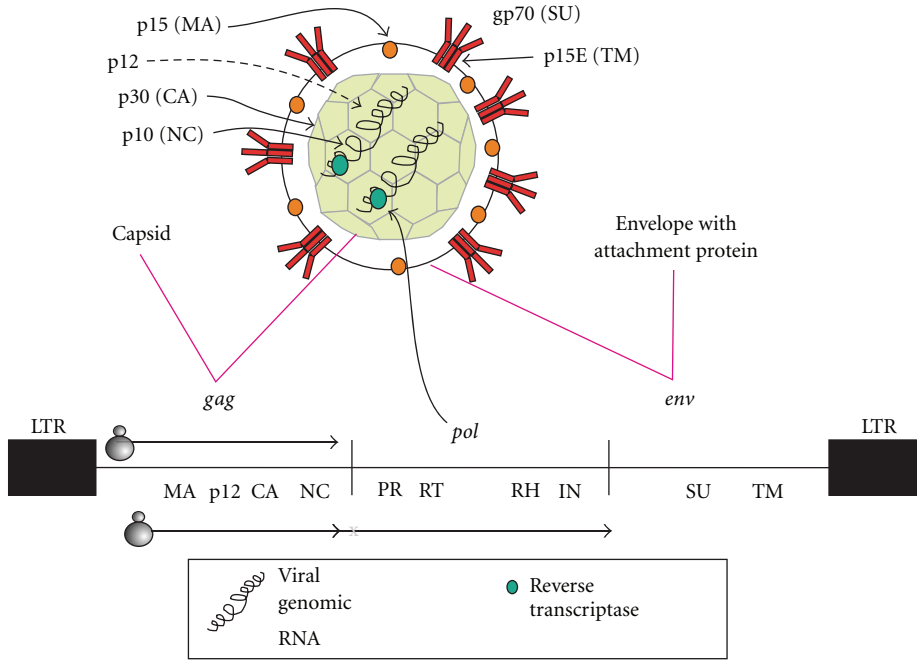


Figure 2: Structure and genome of a gammaretrovirus. The nucleocapsid is built from a hexameric lattice [46]. MA: matrix (p15), CA: capsid (p30), NC: nucleocapsid (p10), PR: protease, RT: reverse transcriptase (shown as a green dot), RH: RNAse H, IN: integrase, SU: surface unit (gp70), and TM: transmembrane protein (p15E). P12 is a small protein encoded from the portion between p15 and p30 in *gag*. LTR: long terminal repeat. The translation of glycoGag and normal Gag is indicated by the respective ribosome symbols. A Gag-Pol polyprotein is occasionally produced by suppression of a stop codon at the end of *gag*.

taxonomy and will be reported in a more complete form in future papers. Especially many seemingly intact, potentially infectious MLLV proviruses were found in the mouse. In this review, we will concentrate on MLLV of mice and mention other rodents, pigs, felines, primates, and some marsupials. Of 7646 retroviral sequences detected in the mm8 assembly, 1461 were gammaretrovirus-like [47]. Some of the latter (300 proviruses) scored higher than 2000 by ReTe (Figures 3 and 4). This is a high score, achieved only by complete or virtually complete proviruses. Indeed, they all turned out to be complete proviruses with very few stop or shift (indel) mutations which could incapacitate the virus. As mentioned above, the 300 proviruses included 35 which had no such mutations. They were structurally "intact" by bioinformatic criteria. The 35 had less than 0.5% LTR divergence. They are marked with green arrows in the Pol tree presented in Figure 3. Thus, the 35 proviruses have hallmarks of being infectious and also belong to the most recently integrated murine ERVs.

Three major groups of high scoring murine gammaretroviral proviruses, named gamma 1–3 (G1–G3), were observed.

Group G1 (188 members, 10 with open reading frame (ORF) in *gag, pro, pol,* and *env*) members encompassed the "*Mus musculus* endogenous retrovirus" (MmERV; GenBank Id AC005743 [13], as interpreted by RetroTector online [49]). *Mus dunni* ERV (AF053745) [14] is highly related. The most similar nonmouse viruses were from rat chromosomes 7 and 17 (nr4 assembly), and more distantly, gibbon ape leukemia virus (GaLV, PCGGPE) and koala retrovirus (KoRV, AF151794) sequences.

Group G2 (59 members, 3 with ORF in *gag, pro, pol,* and *env*) contained the GLN retroviruses described by Ribet et al. [15]. It was most related to rat sequences at chromosomes 7 and 9.

A group of porcine ERVs (PERVs) located at chromosomes 9, 10, 12, and 4 (susScr10 assembly) were 74% similar to the consensus of Group G2, and 70% to the consensus of Group G1. MuRRS [18] and MuERVC [19] sequences were ancestral to groups G1 and G2 at the level of 64% similarity to their consensuses.

Group G3 (53 members, 22 with ORF in *gag, pro, pol,* and *env*) encompassed the ampho-, eco-, xeno-, poly-, and modified polytropic MLVs [50]. Most of the MLVs which have been prominent in retrovirological research for half a century are ecotropic [51]. Amphotropic MLVs are primarily exogenous, while the others are mainly endogenous. The recombinant endogenous *Mus spretus* proviruses [16] emerged between modified polytropic and xenotropic proviruses. The HEMV provirus was at the root of the G3 branch [17].

The three major groups were discernible in trees made with several techniques resulting from alignment of nucleotides and protein sequences of the three genes *gag, pol,* and *env*. They represent three evolutionarily recent bursts of gammaretroviral proliferation in the mouse and its immediate progenitors. The third group, which includes the retroviruses reported in the human diseases, prostate cancer

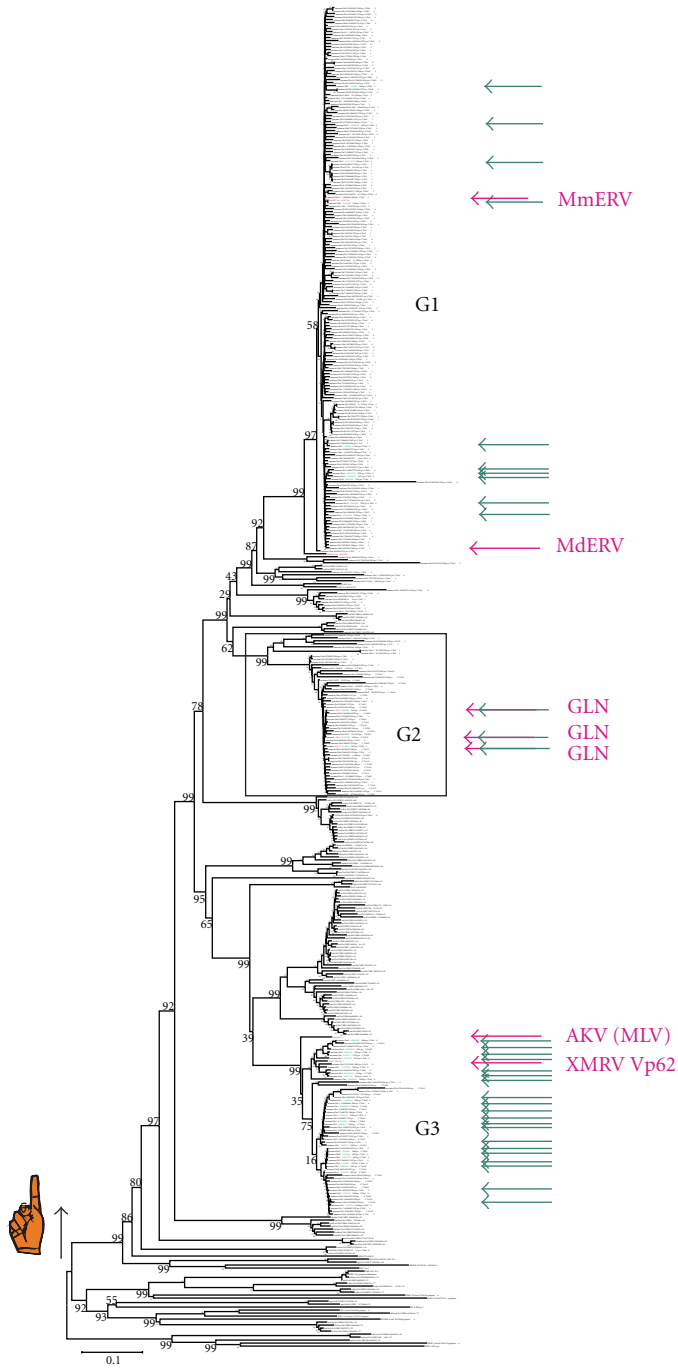


Figure 3: Neighbor-joining (NJ) tree based on Pol amino acid sequences of 300 high ReTe scoring MLLVs, the same as in Figure 4. The three high-scoring murine gamma groups (G1–G3) segregate in a similar way as in a *gag* nt-based tree (Figure 4). Bootstrap values are shown to the left of each branch. Structurally intact proviruses are marked with green arrows. GLN virus [15], MmERV, *Mus dunni* ERV (MdERV), AKV MLV, and XMRV (VP62 clone) are marked with magenta arrows. Murine MLLVs occur from the black arrow in the tree and upwards. The branch labels are either ERV host genus, chromosomal position, ReTe score, provisional genus, "po" for Pol, PBS, and number of stops and frame shifts ("z 0/0" means 0 stops and 0 shifts in *pol*), or a reference Pol name. PBS assignments are made by the 1.01 version of RetroTector. A more complete interpretation of PBS sequences of the G1-G3 groups is given in a forthcoming publication (Elfaitouri et al., accepted in Plos One). The major one zinc finger branch is indicated by the hand symbol. Nonmurine gammaretroviral ERV sequences were taken from the prototype of RetroBank. Further information on which assemblies were used can be found in [47].

and ME/CFS, contains the highest proportion of structurally intact proviruses. It may thus have the greatest zoonotic potential.

In fact, the ancestors of humans were not spared infections with retroviruses related to MLLV. The so-called HERV-T is highly similar to MLLV (Figure 1). It has around 30 representatives in the human genome [52]. More distantly related are ERV-E and ERV-9W. None of the three are structurally intact in the human genome (J.B., unpublished). They are different enough from the murine G3 MLLVs to not interfere with the nucleic acid based methods for XMRV/HMRV detection (J.B., unpublished). Judging from the degree of mutational damage, HERV-T sequences integrated in a human primate progenitor genome around 30 million years ago [52]. MLLVs include the pig endogenous gammaretroviruses, PERV A, B, and C, several of which are infectious and are a problem for xenotransplantation of porcine organs to humans. The murine MLLV groups G1-3 also contain structurally intact proviruses. Very little attention has been paid to groups G1 and G2, while group G3 ("MLVs") has been thoroughly investigated. The number of references regarding the G3 group would be staggering, and out of scope for this review. The receptors and host range of groups G1 and G2 are largely unknown. However, the GLN retroviruses seem to have a tropism similar to ecotropic MLVs [15]. Group G3 is known to contain MLLVs with several envelope-determined tropisms, see, for example, [53–55].

2.2.3. Known and Probable Instances of Transspecies Transfer of Gammaretroviruses. As seen in Figure 5, MLLVs can infect a wide range of hosts. For example, the XPR1 receptor, which is used by xeno- and polytropic MLVs, is common among vertebrates [57, 58]. In some cases, prey-predator relations probably contributed to the transmission [59]. In other cases, there are no such known relations. The wide range of hosts is reflected in the panorama of their receptors [55, 57, 58, 60–65]. They are known to spread via several modes: often via saliva (e.g., into wounds of fighting animals) and sexual contact [26, 62, 66–72]. Moreover, chimpanzees seem to have been infected with MLLVs from baboon and other primates [59], while baboons and cats also have common MLLVs [73]. Thus, MLLVs and similar gammaretroviruses have a tendency to spread between vertebrates.

However, a barrier against spread to humans may be the strong anti-α-galactosyl antibodies in humans, which can neutralize viruses coming from species with different glycosylation patterns, like the mouse [74]. Once the virus has entered the body, its sugars will follow the human glycosylation pattern, and the virus will not any longer be neutralized. Therefore, this barrier is not absolute.

A variety of other restrictions, like the APOBEC cytidine deaminases [50, 75, 76], tetherin [53], and TRIMs [77–79] also affect retroviral spread between species. However, restrictions may be as important within a natural host as between hosts [80, 81]. The high XMRV replication in the cell line 22Rv1 [82] and the ready growth of XMRV in LNCap cells [83], both RNAse L-deficient human prostate cancer cell lines, plus the ability to grow in human PBMCs [84], indicate the ability of XMRV to grow in human cells [84] and the importance of an intact interferon system for the defence against it. These barriers to spread could probably be overcome, and humans be infected by XMRV, although the infectivity *in vivo* is hard to predict. It was recently reported that XMRV can grow in human PBMCs [84].

Judging from the wide spread of MLLVs, a zoonotic spread of XMRV/HMRV from mouse to human, directly or indirectly via another vertebrate, is not impossible. Humans are occasionally exposed to animals which harbor MLLVs. For example, microbes known to spread to humans from pets are viruses (arena-, hanta-, pox-, orthomyxo-, and rhabdoviruses), bacteria (chlamydiae, salmonella, tularemia, and leptospira), protozoa (toxoplasmosis), and helminths (worms). Rabbits, mice, rats, and guinea pigs are frequent as pets. The frequency of animal contact should therefore be recorded in epidemiological investigations regarding MLLVs, like XMRV/HMRV, in humans.

Like other MLLVs, a human MLLV would be expected to spread via kissing, sex, intravenous drug use, blood donation, and possibly via breast feeding. Enough systematic tests for MLLVs in the corresponding body fluids have not been performed. There should also be an overrepresentation of XMRV/HMRV in intravenous drug users and in patients infected with other sexually transmitted microbes, like HIV [85, 86]. This needs more study. Credible transmission chains between ME/CFS patients (with the exception of outbreaks), between PC patients, from ME/CFS to PC, and from PC to ME/CFS have not been reported (cf. Table 1).

3. Did MLLV Spread Zoonotically to Humans?

Gammaretroviruses related to the MLV were found 2006 in a few percent of patients suffering from prostate cancer [9]. They were initially named XMRV. In 2009, XMRV was also found in patients suffering from ME/CFS [6]. In 2010, the term XMRV was replaced with HMRV, because gammaretroviral sequences found in ME/CFS were found to be more diverse than just XMRV [56].

3.1. The Findings in PC. In 2006, Urisman et al. reported the discovery of a novel retrovirus in a subpopulation of prostate cancer patients in the United States [1, 8, 9]. Using fluorescent in situ hybridization, the viral nucleic acid was located to stroma cells, not the cancer cells. However, others found it also in the cancer cells [110]. This retrovirus was identified by means of a DNA microarray ("Virochip" [8, 9, 111]) screening of known cancer samples. The DNA microarray contained 11 000 pieces of 70 bp long oligonucleotides from approximately 950 evolutionarily conserved viral genome sequences. It has been used to screen for the presence of viral DNA and also identify which family the detected virus belongs to. Its success is a practical demonstration of the utility of a phylogenetically directed search for new viruses. The prostate cancer results are covered by other reviews in this volume. Only selected data will be discussed here.

3.1.1. If XMRV/HMRV Is Not Situated in Cancer Cells, How Could it Contribute to PC? MLLVs are not associated with prostate cancer in animals. Therefore, the existence of

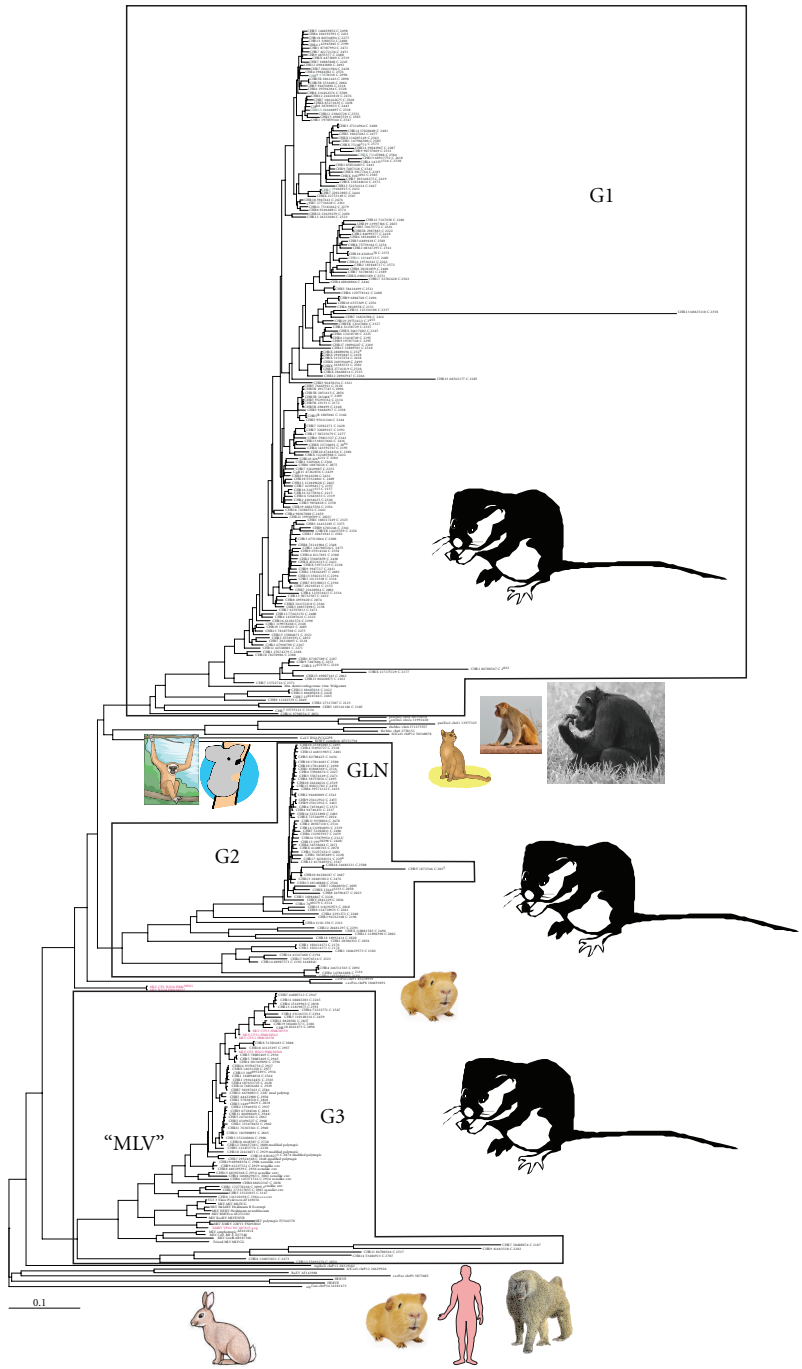


Figure 4: *gag* sequences of 300 high scoring mouse gammaretroviral sequences were aligned together with reference sequences. MLV sequences with ascribed tropism, from GenBank, were also added. The tree was rooted with a divergent rabbit gammaretrovirus-like sequence from scaffold 34, position 34101473 (oryCun1 assembly). Sequences in red ("HMRV") are from the paper of Lo et al. [56]. They were from ME/CFS patients ("CFS") or blood donors ("BD"). Two blood donor sequences from the Lo et al. study came out at the base of group G2, in other trees (not shown) at the base of group G3. The other emerged in group G3. The branch labels are either ERV host genus, chromosomal position, provisional genus ("C": gamma), and ReTe score, or a reference Pol name. Genomic ERV sequences taken from the prototype of RetroBank were named as oryCun: rabbit, cavPor: guinea pig, felCat: cat, panTro: chimpanzee, or rheMac: rhesus macaque. Mouse sequences are just shown with their chromosomal location.

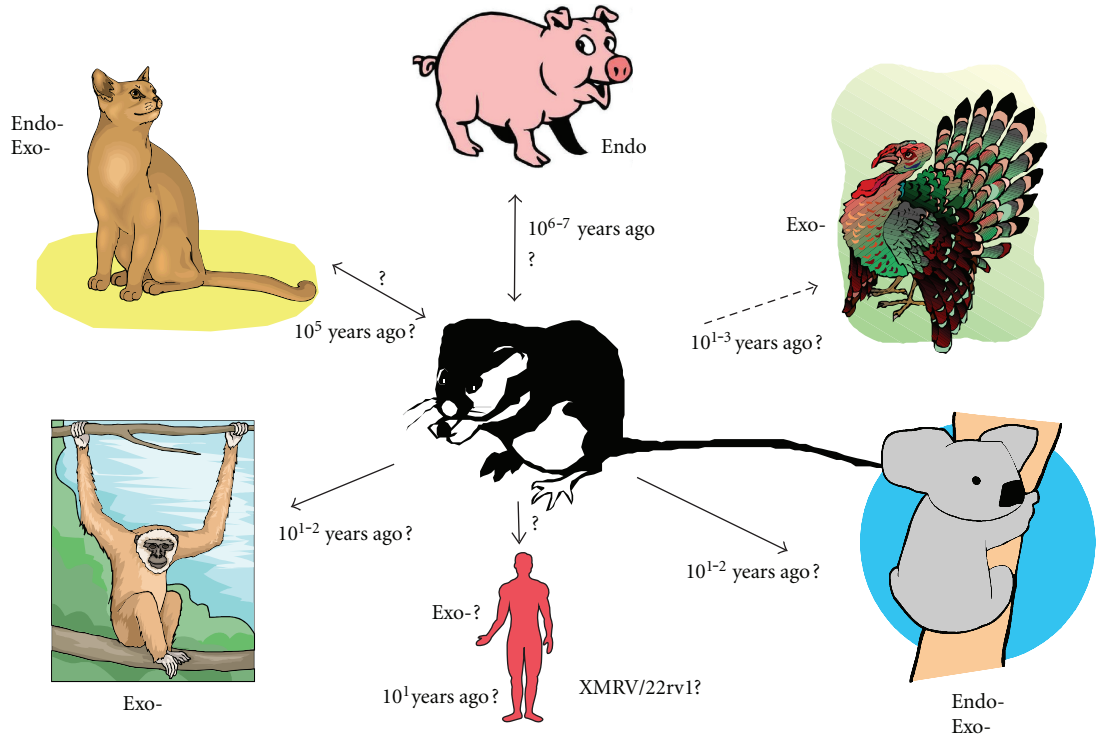


Figure 5: MLLVs have spread among vertebrates in recent evolutionary time. The approximate time estimates to the last common MLLV progenitor are based on references given in the text, and on the phylogenetic analysis of Figure 1. Some gibbon apes in captivity have gibbon ape leukemia virus (GaLV). Koalas have recently been infected with koala retrovirus (KoRV). More distant relatives of the murine MLVs occur in pigs and cats. Porcine MLLV ERVs (PERVs) are MLLVs but the interspecies transmission routes are uncertain. Cats have several endogenous and exogenous MLLVs, including feline leukemia virus (FeLV). Birds have recently been infected with reticuloendotheliosis virus (REV). REV is not strictly an MLLV, but is a gammaretrovirus highly related to MLLVs. Its origin is uncertain, but its closest relatives are the ERV-T-like proviruses from opossum and primates. Humans may also recently have been infected with murine MLLVs, namely XMRV, although there are now indications that this is a laboratory contamination with the 22Rv1 virus. Endo-: endogenous retrovirus. Exo-: exogenous retrovirus.

an MLLV in human prostate cancer is unexpected. Initial reports indicated that the retrovirus was integrated into stromal cell fibroblasts in the prostate cancer samples. This could present a problem for the hypothesis that the newly discovered virus is part of the oncogenic process. However, it is generally accepted that there are close interactions between the epithelial cells and the underlying stromal cells, such as fibroblasts, smooth muscle cells, endothelial cells in blood vessels, and pericytes, both in normal tissue and in tumours [112, 113]. This interaction consists of both paracrine signalling and interactions with the extracellular matrix, which *in vivo* have been shown to have a significant impact on the growth of transformed cells. It was shown 1966 that polyoma-infected epithelial and stromal cells would not transform unless grown in contact with the underlying stroma [114, 115]. It has also been shown in both *in vivo* and *in vitro* studies that tumour-associated fibroblasts contribute to the transformation of immortalized epithelial cells [116], indicating that there are permanent changes in the stromal cells in tumours. What is even more interesting is that it has been shown that the tumour-associated fibroblasts can cause nontumourigenic prostate epithelial cell lines to transform if cocultured [117]. Thus, one cannot dismiss an MLLV as being uninteresting with regard to tumourigenesis in the prostate.

3.2. The Findings in ME/CFS. The main symptom of CFS is a persistent and debilitating fatigue with rapid onset. It often commences after an episode of influenza-like symptoms. The afflicted patients were previously healthy [87]. The symptoms include fatigue, loss of memory or concentration, sore throat, painful lymphadenopathy, muscle pain, headache, unrefreshing sleep, and extreme exhaustion after exercise. The cause of CFS is not yet known. It is in need of clinical and laboratory studies for its further definition. ME/CFS can, however, be diagnosed according to internationally accepted clinical criteria, see, for example, [118]. It seems to be a rather common disease, maybe amounting to 0.5% of the population [118]. Finding the cause, new diagnostic techniques, and, hopefully, a cure, for this often debilitating disease is a high medical priority. ME/CFS borders to the diseases fibromyalgia (FM) and irritable bowel syndrome (IBS). FM-like chronic pain and IBS-like intestinal symptoms are common in ME/CFS [119].

In 2009, Lombardi et al., in Judy Mikovits' group, reported the discovery of the XMRV virus, using PCR and

Table 1: How well does XMRV/HMRV fulfill the expected properties of an MLV spread to humans + means an argument for, and—an argument against expectation?

Property	Expected finding	Observed	Conclusion, for or against XMRV/HMRV in humans?
Discovery/followup	Viral nucleic acid	+Hybridization array w. conserved viral 70-mers, an insensitive technique [9]. +Serendipitous.	For?
		+PCR (positive, XMRV [87], positive, HMRV [56] and −negative [3, 85, 88–100] results). −Proven or suspected contamination w 22Rv1-like virus and mouse DNA [3, 57, 93–96, 99–101].	Against?
		+Integration into host genome [8]. −Some of the integration sites occur in an XMRV-infected cell line [99].	Against?
		+Cloning of XMRV into infectious clone (PC, ME/CFS) [8]. −High sequence similarity and unique recombination suggest contamination with DNA from XMRV producing cell line [2].	Against?
	Virus isolation	+Virus isolation (ME/CFS patients) [6, 102, 103]. −High sequence similarity, lack of evolution, and unique recombination suggests contamination from XMRV producing cell line [2, 82]. −Isolation results cannot be reproduced [3].	Against?
	Antiviral immune response	+SFFV FACS (ME/CFS) concordance with PCR outcome [6, 102], +ELISA (PC, ME/CFS), positive [104] and −negative [84, 92, 105–107] outcomes, −Virus neutralization, negative outcome [90], −Western blot (ME/CFS) negative outcome [105, 108], −CMIA (ME/CFS), negative outcome [109], −T-cell response, negative outcome [86].	Against?
Epidemiology, early phase	Contact with mouse	Not observed/reported	Against?
Epidemiology, late phase	Human-human spread, via saliva, sex, and mother-child	−Although occasional ME/CFS outbreaks occur, most cases are sporadic, with no spread between spouses. −No reported epidemiological link between prostate cancer and ME/CFS −No known overrepresentation in STD and in IVDU [85].	Against?
Pathogenesis	Leukemia, lymphoma	Prostate cancer?	For or against?
	Immunodeficiency	Immunodeficiency?	
	Encephalitis (MAIDS)	Myalgic encephalomyelitis?	
	Enteritis (MAIDS, FAIDS)	Irritable bowel syndrome?	
	Autoimmunity	Thyroiditis?	
Replication	Relatively easy to detect in blood	Hard to detect in blood.	Against?
Immune response	Strong B-cell response, positive WB	Weak or absent B-cell response.	Against?

serology, in 67% of chronic fatigue syndrome (ME/CFS) patients compared to 3.7% in healthy controls [6]. Reports which verify [56, 102, 103] and do not verify [84, 88–93, 105, 106, 108, 120] the original ME/CFS report have come. The situation is volatile and cannot be extensively covered here. The conflicting results may be due to methodological differences, an uneven geographic distribution of XMRV/HMRVs, or viral genetic variation. Switzer et al. used a Western blot with a lysate from MLV, another group G3 virus, to examine antibody response in 51 CFS patients and 53 healthy donors. No specific reaction was seen [105].

In a UK study involving 170 CFS patients as well as 395 non-CFS patient controls, 4.6% of the samples contained neutralizing antibodies, but only one of these was from a CFS patient. Most of these positive sera were able to neutralize MLV virus particles pseudotyped with Env-proteins of other viruses indicating significant cross reactivity [90]. Hohn et al. searched for antibody activity of 36 CFS and 112 MS patients and 27 healthy controls using an Env-ELISA and a capture Gag-ELISA. No evidence of specific seroreactivity was found [84].

3.2.1. Could an MLLV Cause the Combination of Neuro- and Immunopathology Which Occurs in ME/CFS? MLLV infections are sometimes associated with immunosuppression [121]. ME/CFS patients have a degree of immunosuppression, neurological symptoms, and often an enteritis. Giardia has been associated with ME/CFS [122, 123]. Mice with MAIDS are especially sensitive to intestinal parasites like Giardia, a similarity which may be fortuitous [122, 123]. Likewise, cats with FAIDS are also immunosuppressed and develop enteritis. Although such correlations are diffuse and may be spurious they indicate that infectious agents could give ME/CFS-like disease.

3.3. Other Diseases Expected to Occur If There Is an Infectious Human Gammaretrovirus. Murine and feline diseases where MLLVs are known to play a major role are leukemia, lymphoma and encephalitis (and other neurological diseases) and, as mentioned, immunodeficiency [124–132]. Autoimmune disease has also been linked to MLLVs [133, 134]. MLLVs should thus be searched for in these diseases. So far, studies of associations of XMRV/HMRV with such human diseases have not been published.

3.4. Can Phylogeny Direct Our Efforts to Detect MLLV Infection in Humans?

3.4.1. Viral Nucleic Acid Detection. Nested PCRs have been used in several of the positive XMRV reports. Such PCRs have a high sensitivity, but also a high risk of amplicon contamination.

The lower sensitivity of microarray analysis makes it less susceptible to contamination. This makes the initial serendipitous observation of XMRV in prostate tissue [9] especially credible. Although PCRs are more sensitive, they may miss imperfectly matching targets. High sensitivity also means high risk of contamination. Amplimers, synthetic sequences, and plasmids containing target sequences are notorious problems. However, also genomic DNA harboring target sequences (ERVs) is a problem. Mouse DNA is a special case, because it contains many HMRV PCR target sequences per copy of mouse genome (see above). Thus, each XMRV/HMRV PCR should be evaluated for its detection range and tendency to give false positive results due to mouse DNA contamination. Several of the PCRs which have been used to detect XMRV react also with mouse DNA. Some, like the integrase-based PCR of Ila Singh [135], do not react with mouse DNA unless the DNA is present in high concentration. Others react strongly with mouse DNA. Mouse DNA contains at least two sequences which are highly similar to XMRV, with ORF in gag, pro, pol and env: Proviruses at chromosome 1, position 172778230 and chromosome 5, position 23221036. The similarity is obvious in *pol* and *env*, less so in *gag* (mouse genome assembly mm8, which comes from C57Black). These proviruses are situated next to XMRV VP62 in the Pol-based tree in Figure 3. All broadly targeted ("HMRV-specific") PCRs react with mouse DNA, while some "XMRV specific," like the Singh PCR, do not react, or react only with high amounts of mouse DNA (Elfaitouri et al., submitted). Among 30 vertebrate genomic DNAs analyzed bioinformatically, mouse DNA is most likely to give such spurious PCR signals (Blomberg and Elfaitouri, unpublished).

A test which lends high credibility to a positive provirus detection is if integration sites in the host genome can be cloned and sequenced. This has been reported for XMRV in PC [7]. However, Garson et al. [99] claimed that integration sites in 2 of 14 prostate cancer patient samples reported from Silverman's group [8, 9] were identical to those of the experimentally infected human tumour cell line DU145 used in the same laboratory. This raises the possibility that this finding is due to contamination.

The Problem of Mouse DNA and XMRV DNA Contamination. Where Does It Come from? Several laboratories have reported frequent occurrence of mouse DNA in samples from humans. Some of the PCRs used for detection of XMRV/HMRV also become positive when mouse DNA is present, because (as explained earlier in this review) the mouse genome contains many proviruses which react in these PCRs. However, some XMRV PCRs do not react with mouse DNA [110]. They should be less likely to give false positive signals due to presence of mouse DNA. Presence of mouse DNA in human samples may sound absurd and is often not easy to explain. It may be caused by contamination of chemicals and biologicals used to prepare the samples. For example, mouse DNA may be present on microtomes used for preparation of tissue sections from both mouse and humans. Further, some cell lines (especially hybridomas of mouse origin) contain high amounts of XMRV or related MLVs and can contaminate other cell lines [136, 137]. It is therefore logical that murine monoclonal antibodies, including anti-Taq polymerase antibodies, which are used to provide a "hot" start in PCRs, sometimes contain traces of nucleic acid from MLVs [101]. Moreover, the highly XMRV producing cell line 22Rv1 contains a virus which is very similar (essentially identical; see Figure 4) to reported XMRVs [99]. It is a human prostate cancer cell line which was infected with XMRV during passage in nude mice [2]. According to recent information, XMRV arose by a unique recombination event between two defective MLV sequences [2]. It is therefore reasonable to assume that XMRV originated from the 22Rv1 virus which then has been a contamination source of many of the published positive results from prostate cancer and ME/CFS. In fact, all cell lines which have been passed in nude or SCID mice should be suspected of retroviral contamination.

If contamination explains all positive PCR results in ME/CFS and prostate cancer, why would the frequency in ME/CFS patients be at least tenfold higher than in the controls? The patient samples may have been more frequently opened than the control samples. At least two kinds of contamination, with mouse and XMRV DNA, respectively, have to be invoked. Although false positive PCR results due to contamination in the laboratory is a frequent event, contamination on such a grand scale is beyond previous experience.

There are similarities between the XMRV/HMRV and the "Human Retrovirus 5" (HRV5) stories [138]. HRV5 is one of the so-called "rumor viruses" [139]. It turned out to be a defective rabbit betaretrovirus (RERV-H) whose DNA is abundant in rabbit sera [140]. The rabbit genome contains around 700 copies of RERV-H [140]. Any laboratory which handles rabbit sera is at risk of RERV-H contamination. In analogy with this, a low level of mouse (or XMRV) DNA could be present in laboratory reagents, cell cultures, or in the laboratory environment, as evidenced by many confirmed reports of contamination, or unconfirmed reports of human retroviruses [73, 93–96, 99–101, 136, 137, 141–165]. Thus, extensive contamination controls must be performed if PCR is used for detection of XMRV/HMRV.

3.4.2. Viral Reverse Transcriptase Detection. Reverse transcriptase (RT) activity is a fundamental and conserved function of retroviruses. Detection of RT activity is an established method for retrovirus discovery and diagnosis. There is an evolutionary limit to the extent to which the enzyme can mutate. Presence of significant retroviral RT activity in a biological sample is thus not only an indication of virus protein expression, but of retrovirus replication. RT activity assays thus give an additional dimension compared to detection of viral nucleic acids or proteins. The RT enzymes from different retroviruses have different enzymatic properties. By varying pH and the composition and concentrations of certain components, it is possible to optimize assays for a virus family or subgroup [166]. RT assays can also be performed with PCR readout, which gives a very high sensitivity [167, 168]. Quantification of RT activity may be complicated by a myriad of RT inhibitory factors and requires enzyme purification. A simple colorimetric RT activity assay [166] can be used both for monitoring propagation of XMRV virus in cell culture and for direct detection in samples from humans [169]. RT assays are somewhat less sensitive than PCR and have less problems with contamination and a broader detection range. An RT assay optimized for XMRV has been developed (http://cavidi.se/). It has a minimum level of detection of 0.01 μU RT activity, which corresponds to approximately 50 virus particles per reaction (Elfaitouri et al, accepted for publication in Plos One). It is currently used as an independent technique for following isolation and propagation of MLV-related viruses in our laboratory.

3.4.3. Virus Isolation. The Mikovits group at the Whittemore Peterson Institute reported a high frequency of virus isolation from ME/CFS patients [6, 102, 103]. We are not aware of a report on XMRV isolation from prostate cancer patients. Virus isolation is inherently less susceptible to contamination than PCR is, because retroviruses are easy to disinfect. They also lose infectivity after drying. The virus isolation results are therefore the mainstay of the proponents of XMRV [102, 103]. An especially efficient and specific virus isolation test seems to be the DERSE (detector of exogenous retroviral sequences) assay [170]. The isolation results from ME/CFS patients were recently contested [3]. Patients which earlier were reported to be XMRV isolation positive were found to be negative on retesting. This raises the possibility that also the virus isolation results were due to contamination with 22Rv1 tissue culture virus. The 22Rv1 virus is present in billions of copies per mL of tissue culture supernatant. Retroviral contamination of cell cultures is common, see, for example, [136, 137, 171]. Low or moderate expression of potentially infectious ERVs in cell culture is a particularly vexing problem [137, 143, 172–176].

3.4.4. Serology. Antigens for use in XMRV/HMRV serology range from synthetic peptides to recombinant proteins. In this situation, cross-reactive epitopes in Gag and transmembrane proteins are of special interest. Epitopes from, primarily, Env and Gag proteins of XMRV and related MLVs should be covered. It is fortunate that much information regarding B-cell epitopes is available for MLVs and FeLVs (against which an effective vaccine exists). It can guide the selection of synthetic peptides. Synthetic peptides, primarily mimicking linear epitopes, have been useful for development of serological assays for detection of other retroviruses such as HIV and HTLV [177–182]. However, an optimal serological assay should cover both linear and conformational epitopes. Whole virus [105, 109, 183, 184], recombinant XMRV proteins [84, 104, 106, 109, 183, 185], and a "surrogate" spleen focus forming virus (SFFV) fluorescence-activated cell sorter (FACS) antibody test [6] have been used. SFFV has an envelope which is a recombinant between the envelopes of the infectious Friend helper virus and an endogenous polytropic virus. It also has a large deletion involving the SU/TM cleavage site. Cells with and without transduced SFFV envelope are incubated with serum and fluorescent antihuman IgG and then run in an FACS. This is an elegant and specific technique, but is dependent on a few cross-reactive envelope epitopes. Western blot (WB) using whole virus (or a set of recombinant proteins) [105] is a *de facto* serological golden standard in clinical retrovirology. It would be desirable to have a confirmatory WB assay for serological XMRV diagnosis. Screening assays could be existing enzyme immunoassays (EIAs), suspension arrays [120], or chemiluminescent magnetic microparticle immunoassays (CMIA) [109]. Neutralization assays [89] can be highly specific, but may be too narrow if a broad search is desired. The FACS analyses of antibodies binding to SFFV envelope transduced cells should also be very specific. None of the latter two are, however, suitable for large-scale screening. There is a long history of serological findings of gammaretrovirus antibodies and antigens in human disease [73, 186–203]. Given the tendency of serological methods to cross-react, weak serological reactions to a few epitopes alone are not strong evidence.

A serological diagnosis of a retroviral infection needs demonstration of an immune response at several epitopes. It cannot be the only serological evidence. Ideally, several tests with different specificities, covering both broadly cross-reactive and specific, linear and conformational, epitopes should be used. Several of these serological assays are capable of detecting antibody reactivity to other MLVs. Thus, although it may not be intentional, the intense hunt for XMRV antibodies is also a hunt for other MLLVs in humans. The largely negative outcome [84, 105, 106, 108, 204] may be taken as evidence against widespread MLLV infection in humans.

3.4.5. Why Was XMRV/HMRV Not Discovered in the Virus Cancer Program during 1964–1977? A considerable US effort to find new retroviruses was the Virus Cancer program [205]. Virus isolation and reverse transcriptase assays were main techniques. MLV and similar viruses are relatively easy to cultivate. They were known at the time and were also main targets for the program. It is therefore notable that such a virus was not detected in humans with cancer during this project. Reasons could be manifold, either due to bad luck, inappropriate methods, due to an introduction of XMRV/HMRV into humans, or spread of a tissue culture contaminating virus after the conclusion of the Virus Cancer program.

3.4.6. Summary of Current Controversies Regarding XMRV/HMRV. Since the initial reports of XMRV in US patients with prostate cancer and CFS, several research groups have been attempting to replicate these results. Especially, the connection of XMRV to CSF has raised considerable interest in this virus. However, it has not been possible to detect the virus in studies from China where Hong et al. have analyzed samples from 65 CFS patients and 85 healthy controls [92], from Netherlands where van Kuppeveld et al investigated samples from 76 CFS patients and 69 matched controls [98], from UK where Groom et al. tested samples from 170 CFS patients and 395 non-CFS control patients [90], and Erlwein et al. investigated samples from 186 CFS patients [89], and from the US where Switzer et al. analyzed samples from 51 CFS patients and 53 healthy controls [105], using either PCR-based methods for detecting viral RNA/DNA or methods for detecting neutralizing antibodies against XMRV. Hohn et al. were likewise unable to detect XMRV in any of the 589 German prostate cancer samples they analyzed for the presence of XMRV [84, 106]. To further confuse the issue, a US research group failed to detect the XMRV itself but was able to detect viral sequences closer related to other MERVs ("HMRV"; here shown to belong to group G3 MLLV) in a retrospective study of blood samples from CSF patients [56].

3.4.7. An Attempt to Reconcile Current Results Regarding XMRV/HMRV with Each Other.

Why Is XMRV/HMRV So Hard to Detect by PCR and Serology? A possibility which could reconciliate the conflicting findings could be that a chronic XMRV/HMRV infection becomes progressively harder to detect both by nucleic acid, virus isolation, and serological methods. XMRV/HMRV would then establish a low-grade infection in a limited number of cell types, with a waning immune response. This is reminiscent of what was seen in experimentally XMRV-infected macaques [109, 183]. The dynamics of antibody response elicited by XMRV were studied in five XMRV-infected macaques. Using recombinant gp70, p15E, and p30 in western blots and CMIA, Qiu et al. found evidence of antibodies two weeks after infection. The antibodies persisted for at least 158 days. Although all three proteins elicited an immune response, antibodies to recombinant gp70 and p15E showed higher sensitivity than p30 [109, 183]. The Western blots were very clear. Such XMRV Western blots were never reported from humans. There was a tendency for the antibody levels to decrease over time. Stimulation with a dose of XMRV antigen gave rise to a burst of viral replication and a rise in XMRV antibodies. In another study, *Mus pahari* was experimentally infected with XMRV [184]. Antibodies to XMRV Env (p15E and gp70) and Gag (p30) were detected in Western blots and in ELISA tests. Neutralizing antibodies also developed.

Thus, the expected course of an XMRV/HMRV infection is an initial phase with intense viral replication, easily detected by PCRs on nucleic acids from plasma or PBMCs, followed by development of a strong antibody response which can be demonstrated by several kinds of serology, and a full WB pattern, similar to the situation during HIV infection.

However, the difficulties of finding both viral nucleic acid in blood samples and a weak or nonexistent antibody response in virus isolation or XMRV/HMRV PCR-positive persons lead us to two alternate interpretations.

First, we must hypothetically consider a model of low-grade chronic XMRV/HMRV infection of humans. Such a "stealth" infection could have similarities with ERVs. The immune response to ERVs is abnormal, possibly because they are perceived as "self" by the adaptive immune system [206]. Their protein expression may also be weak. In putative "stealth" non-ERV RV infection a low degree of continuous antigenic stimulation could lead to a low, and waning immune response [207]. Although a vigorous antibody response is the most common reaction to a retrovirus infection, a very weak antibody response is also seen in some cases of HLTV-2 infection, which can also be accompanied by a low degree of viral replication [208, 209]. It is a considerable difficulty for the diagnosis of HTLV-2 infections in humans by PCR and serology. Likewise, HIV patients which were treated early during the infection may develop an abortive immune response [210, 211]. It is conceivable that the XMRV/HMRV situation could be similar. There are aspects of the MAIDS/FAIDS models which fit with ME/CFS and this model.

The second alternative is that all reports of XMRV/HMRV in humans have been due to contamination or serological cross-reaction. The PCRs could have been confounded by various forms of contamination (see below). The positive serologies in ME/CFS patients have largely been from the surrogate SFFV FACS antibody test [6], which alone

does not fully suffice as evidence. It would be a sad outcome of a fascinating and important story.

3.5. Medical and Ethical Consequences of the Uncertain Diagnostic Situation. The finding of XMRV/HMRV in ME/CFS has far-reaching implications, for the personal life of the patients (sex, kissing, breast feeding, etc.), for the development of diagnostic methods, for transfusion safety, and for the understanding of other human diseases with a possible retroviral etiology. It is reasonable to demand that measures to protect blood transfusion recipients from infection are as rigorous as the sensitivity and specificity of available tests allow. In the situation of today, where the reported frequency of XMRV infection found with different methods in blood donors or comparable healthy individuals varies from 0 to 7%, there is no simple test strategy available. However, the mere suspicion that a retrovirus like XMRV is frequent in patients suffering from ME/CFS is a basis for abstaining from using such patients as blood donors [212]. The finding of XMRV in PC also raises medical and ethical questions. However, the frequency of positivity is at a few percent, and claims of a connection have not reached the high frequencies reported in ME/CFS (>60%).

Both ME/CFS and PC patients suffer from the uncertainty regarding XMRV/HMRV positivity in the two diseases. The patients must make personal decisions regarding sexual contacts, kissing, and breast milk feeding of their children. Few PC patients will know their alleged XMRV/HMRV status, but the rather widespread testing of ME/CFS patients for this virus has created a group of patients who are left in limbo. The temptation to start antiretroviral treatment [213] despite the current scientific controversy can be hard to resist.

4. Conclusions

Research on XMRV/HMRV in humans is evolving rapidly. There is a great need for confirmation of the reports on XMRV/HMRVs in PC and ME/CFS. In view of the recently demonstrated diversity of retroviral sequences in ME/CFS, it is also important to establish the detection range of XMRV/HMRV detection methods. Contamination of cell cultures with 22Rv1 virus and PCRs with MLV nucleic acid and mouse DNA is known to occur. Whether all reports on MLLVs in humans can be explained by them is uncertain, but not unlikely. The XMRV/HMRV story has both credible and less credible aspects (Table 1). The original XMRV detection in prostate cancer was serendipitous and made with several independent techniques, together forming a credible case. The proven integration into human DNA was especially convincing. The finding of XMRV/HMRV in ME/CFS also has a credible aspect; the immunomodulating properties of MLLVs could theoretically explain the disease. However, the epidemiology of XMRV/HMRV transmission still is unclear. The absence of an easily measurable immune response is also a memento.

We conclude that MLLVs are widespread as ERVs among vertebrates. There are many signs of interspecies transmission of MLLVs. However, only a few of the MLLVs are structurally intact. The mouse genome is unique in its high content of MLLVs. It contains three major MLLV groups, of which two (G1 and G2) have not hitherto been reported. Group G3 contains the MLVs and all or nearly all of the MLV-like retroviruses which have so far been detected in humans, that is, XMRV and HMRV.

The study of XMRV/HMRV is important from a range of perspectives, one of which is screening of blood donors for potentially harmful pathogens. Xenotropic viruses also raise concerns regarding research into xenotransplantation of organs [16].

The detection of human infection with XMRV/HMRV has proven to be difficult. This may either be due to absence of the virus or to a low-grade infection, with a minimal viral replication and a minimal serological response. Although that goes contrary to expectations, such a situation sometimes occurs in HTLV and HIV infections.

Acknowledgments

The financial support from ME Research UK (Charity no. SC 036942), the Irish ME Trust, and Stiftelsen Lars Hiertas Minne (FO2010-0672) is gratefully acknowledged.

References

[1] K. Lee and K. S. Jones, "The path well traveled: using mammalian retroviruses to guide research on XMRV," *Molecular Interventions*, vol. 10, no. 1, pp. 20–24, 2010.

[2] T. Paprotka, K. A. Delviks-Frankenberry, O. Cingoz et al., "Recombinant origin of the retrovirus XMRV," *Science*, vol. 333, no. 6038, pp. 97–101, 2011.

[3] K. Knox, D. Carrigan, G. Simmons et al., "No evidence of murine-like gammaretroviruses in CFS patients previously identified as XMRV-infected," *Science*, vol. 333, no. 6038, pp. 94–97, 2011.

[4] R. H. Silverman, C. Nguyen, C. J. Weight, and E. A. Klein, "The human retrovirus XMRV in prostate cancer and chronic fatigue syndrome," *Nature Reviews Urology*, vol. 7, no. 7, pp. 392–402, 2010.

[5] H. G. Klein, R. Y. Dodd, F. B. Hollinger et al., "Xenotropic murine leukemia virus-related virus (XMRV) and blood transfusion: report of the AABB interorganizational XMRV task force," *Transfusion*, vol. 51, no. 3, pp. 654–661, 2011.

[6] V. C. Lombardi, F. W. Ruscetti, J. D. Gupta et al., "Detection of an infectious retrovirus, XMRV, in blood cells of patients with chronic fatigue syndrome," *Science*, vol. 326, no. 5952, pp. 585–589, 2009.

[7] S. Kim, N. Kim, B. Dong et al., "Integration site preference of xenotropic murine leukemia virus-related virus, a new human retrovirus associated with prostate cancer," *Journal of Virology*, vol. 82, no. 20, pp. 9964–9977, 2008.

[8] B. Dong, S. Kim, S. Hong et al., "An infectious retrovirus susceptible to an IFN antiviral pathway from human prostate tumors," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 5, pp. 1655–1660, 2007.

[9] A. Urisman, R. J. Molinaro, N. Fischer et al., "Identification of a novel gammaretrovirus in prostate tumors of patients homozygous for R462Q RNASEL variant," *PLoS Pathogens*, vol. 2, no. 3, article e25, 2006.

[10] J. Denner, "Detection of a gammaretrovirus, XMRV, in the human population: open questions and implications for xenotransplantation," *Retrovirology*, vol. 7, article 16, 2010.

[11] P. Schmidt, A. Forsman, G. Andersson, J. Blomberg, and O. Korsgren, "Pig islet xenotransplantation: activation of porcine endogenous retrovirus in the immediate post-transplantation period," *Xenotransplantation*, vol. 12, no. 6, pp. 450–456, 2005.

[12] P. Schmidt, G. Andersson, J. Blomberg, A. Malmsten, and O. Korsgren, "Possible transmission of zoonoses in xenotransplantation: porcine endogenous retroviruses (PERVs) from an immunological point of view," *Acta Veterinaria Scandinavica*, vol. 99, supplement, pp. 27–34, 2004.

[13] L. Bromham, F. Clark, and J. J. McKee, "Discovery of a novel murine type C retrovirus by data mining," *Journal of Virology*, vol. 75, no. 6, pp. 3053–3057, 2001.

[14] G. Wolgamot, L. Bonham, and A. D. Miller, "Sequence analysis of Mus dunni endogenous virus reveals a hybrid VL30/gibbon ape leukemia virus-like structure and a distinct envelope," *Journal of Virology*, vol. 72, no. 9, pp. 7459–7466, 1998.

[15] D. Ribet, F. Harper, C. Esnault, G. Pierron, and T. Heidmann, "The GLN family of murine endogenous retroviruses contains an element competent for infectious viral particle formation," *Journal of Virology*, vol. 82, no. 9, pp. 4413–4419, 2008.

[16] Y. T. Jung, T. Wu, and C. A. Kozak, "Characterization of recombinant nonecotropic murine leukemia viruses from the wild mouse species mus spretus," *Journal of Virology*, vol. 77, no. 23, pp. 12773–12781, 2003.

[17] C. H. Tipper, C. E. Bencsics, and J. M. Coffin, "Characterization of Hortulanus endogenous murine leukemia virus, an endogenous provirus that encodes an infectious murine leukemia virus of a novel subgroup," *Journal of Virology*, vol. 79, no. 13, pp. 8316–8329, 2005.

[18] M. Schmidt, T. Wirth, B. Kröger, and I. Horak, "Structure and genomic organization of a new family of murine retrovirus-related DNA sequences (muRRS)," *Nucleic Acids Research*, vol. 13, no. 10, pp. 3461–3470, 1985.

[19] Y. Zhao, C. P. D. Jacobs, L. Wang, and S. C. Hardies, "MuERVC: a new family of murine retrovirus-related repetitive sequences and its relationship to previously known families," *Mammalian Genome*, vol. 10, no. 5, pp. 477–481, 1999.

[20] J. M. Yi, T. H. Kim, J. W. Huh et al., "Human endogenous retroviral elements belonging to the HERV-S family from human tissues, cancer cells, and primates: expression, structure, phylogeny and evolution," *Gene*, vol. 342, no. 2, pp. 283–292, 2004.

[21] J. Blomberg, F. Benachenhou, V. Blikstad, G. Sperber, and J. Mayer, "Classification and nomenclature of endogenous retroviral sequences (ERVs). Problems and recommendations," *Gene*, vol. 448, no. 2, pp. 115–123, 2009.

[22] A. L. Roca, J. Pecon-Slattery, and S. J. O'Brien, "Genomically intact endogenous feline leukemia viruses of recent origin," *Journal of Virology*, vol. 78, no. 8, pp. 4370–4375, 2004.

[23] V. N. Kewalramani, A. T. Panganiban, and M. Emerman, "Spleen necrosis virus, an avian immunosuppressive retrovirus, shares a receptor with the type D simian retroviruses," *Journal of Virology*, vol. 66, no. 5, pp. 3026–3031, 1992.

[24] T. G. Kawakami, G. V. Kollias Jr., and C. Holmberg, "Oncogenicity of gibbon type-C myelogenous leukemia virus," *International Journal of Cancer*, vol. 25, no. 5, pp. 641–646, 1980.

[25] T. G. Kawakami, L. Sun, and T. S. McDowell, "Natural transmission of gibbon leukemia virus," *Journal of the National Cancer Institute*, vol. 61, no. 4, pp. 1113–1115, 1978.

[26] R. Tarlinton, J. Meers, and P. Young, "Endogenous retroviruses: biology and evolution of the endogenous koala retrovirus," *Cellular and Molecular Life Sciences*, vol. 65, no. 21, pp. 3413–3421, 2008.

[27] J. Martin, E. Herniou, J. Cook, R. W. O'Neill, and M. Tristem, "Interclass transmission and phyletic host tracking in murine leukemia virus-related retroviruses," *Journal of Virology*, vol. 73, no. 3, pp. 2442–2449, 1999.

[28] J. M. Coffin, S. H. Hughes, and H. E. Varmus, Eds., *Retroviruses*, Cold Spring Harbor Laboratory Press, New York, NY, USA, 1997.

[29] R. J. Gorelick, D. J. Chabot, D. E. Ott et al., "Genetic analysis of the zinc finger in the moloney murine leukemia virus nucleocapsid domain: replacement of zinc-coordinating residues with other zinc-coordinating residues yields noninfectious particles containing genomic RNA," *Journal of Virology*, vol. 70, no. 4, pp. 2593–2597, 1996.

[30] P. Jern, G. O. Sperber, and J. Blomberg, "Use of endogenous retroviral sequences (ERVs) and structural markers for retroviral phylogenetic inference and taxonomy," *Retrovirology*, vol. 2, article 50, 2005.

[31] D. C. Aziz, Z. Hanna, and P. Jolicoeur, "Severe immunodeficiency disease induced by a defective murine leukaemia virus," *Nature*, vol. 338, no. 6215, pp. 505–508, 1989.

[32] Y. Kubo, Y. Nakagawa, K. Kakimi et al., "Presence of transplantable T-lymphoid cells in C57BL/6 mice infected with murine AIDS virus," *Journal of Virology*, vol. 66, no. 9, pp. 5691–5695, 1992.

[33] C. Simard, M. Huang, and P. Jolicoeur, "Establishment of leukemic T-cell lines from mice inoculated with the MAIDS defective virus," *Virology*, vol. 206, no. 1, pp. 555–563, 1995.

[34] J. Overbaugh, N. Riedel, E. A. Hoover, and J. I. Mullins, "Transduction of endogenous envelope genes by feline leukaemia virus in vitro," *Nature*, vol. 332, no. 6166, pp. 731–734, 1988.

[35] J. L. Rohn, S. R. Gwynn, A. S. Lauring, M. L. Linenberger, and J. Overbaugh, "Viral genetic variation, AIDS, and the multistep nature of carcinogenesis: the feline leukemia virus model," *Leukemia*, vol. 10, no. 12, pp. 1867–1869, 1996.

[36] S. L. Roth, N. Malani, and F. D. Bushman, "Gammaretroviral integration into nucleosomal target DNA in vivo," *Journal of Virology*, vol. 85, no. 14, pp. 7393–7401, 2011.

[37] F. D. Bushman, "Retroviral integration and human gene therapy," *Journal of Clinical Investigation*, vol. 117, no. 8, pp. 2083–2086, 2007.

[38] X. Wu, Y. Li, B. Crise, and S. M. Burgess, "Transcription start regions in the human genome are favored targets for MLV integration," *Science*, vol. 300, no. 5626, pp. 1749–1751, 2003.

[39] S. Hacein-Bey-Abina, C. Von Kalle, M. Schmidt et al., "LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1," *Science*, vol. 302, no. 5644, pp. 415–419, 2003.

[40] C. H. J. Lamers, R. A. Willemsen, P. M. M. L. van Elzakker, J. W. Gratama, and R. Debets, "Gibbon ape leukemia virus poorly replicates in primary human T lymphocytes: implications for safety testing of primary human T lymphocytes transduced with GALV-pseudotyped vectors," *Journal of Immunotherapy*, vol. 32, no. 3, pp. 272–279, 2009.

[41] S. Haraguchi, R. A. Good, G. J. Cianciolo, R. W. Engelman, and N. K. Day, "Immunosuppressive retroviral peptides: immunopathological implications for immunosuppressive influences of retroviral infections," *Journal of Leukocyte Biology*, vol. 61, no. 6, pp. 654–666, 1997.

[42] M. Nelson, D. S. Nelson, G. J. Cianciolo, and R. Snyderman, "Effects of CKS-17, a synthetic retroviral envelope peptide, on cell-mediated immunity in vivo: immunosuppression, immunogenicity, and relation to immunosuppressive tumor products," *Cancer Immunology Immunotherapy*, vol. 30, no. 2, pp. 113–118, 1989.

[43] M. Enserink, "Chronic fatigue syndrome. New XMRV paper looks good, skeptics admit—yet doubts linger," *Science*, vol. 329, no. 5995, p. 1000, 2010.

[44] M. Mangeney and T. Heidmann, "Tumor cells expressing a retroviral envelope escape immune rejection in vivo," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 25, pp. 14920–14925, 1998.

[45] G. Schlecht-Louf, M. Renard, M. Mangeney et al., "Retroviral infection in vivo requires an immune escape virulence factor encrypted in the envelope protein of oncoretroviruses," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 8, pp. 3782–3787, 2010.

[46] M. Yeager, E. M. Wilson-Kubalek, S. G. Weiner, P. O. Brown, and A. Rein, "Supramolecular organization of immature and mature murine leukemia virus revealed by electron cryo-microscopy: implications for retroviral assembly mechanisms," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 13, pp. 7299–7304, 1998.

[47] J. Blomberg, G. Sperber, P. Jern, and F. Benachenhou, "Towards a retrovirus database, retrobank," in *Proceedings of the Centennial Retrovirus Meeting*, R. Daniel, J. Hejnar, A. M. Skalka, and J. Svoboda, Eds., Medimond International Proceedings, pp. 19–22, Prague, Czech Republic, 2010.

[48] G. O. Sperber, T. Airola, P. Jern, and J. Blomberg, "Automated recognition of retroviral sequences in genomic data—RetroTector," *Nucleic Acids Research*, vol. 35, no. 15, pp. 4964–4976, 2007.

[49] G. Sperber, A. Lövgren, N. E. Eriksson, F. Benachenhou, and J. Blomberg, "RetroTector online, a rational tool for analysis of retroviral elements in small and medium size vertebrate genomic sequences," *BMC Bioinformatics*, vol. 10, supplement 6, article S4, 2009.

[50] P. Jern, J. P. Stoye, and J. M. Coffin, "Role of APOBEC3 in genetic diversity among endogenous murine leukemia viruses," *PLoS Genetics*, vol. 3, no. 10, pp. 2014–2022, 2007.

[51] L. M. Albritton, L. Tseng, D. Scadden, and J. M. Cunningham, "A putative murine ecotropic retrovirus receptor gene encodes a multiple membrane-spanning protein and confers susceptibility to virus infection," *Cell*, vol. 57, no. 4, pp. 659–666, 1989.

[52] M. Haltmeier, W. Seifarth, J. Blusch, V. Erfle, R. Hehlmann, and C. Leib-Mosch, "Identification of S71-related human endogenous retroviral sequences with full-length pol genes," *Virology*, vol. 209, no. 2, pp. 550–560, 1995.

[53] C. A. Kozak, "The mouse "xenotropic" gammaretroviruses and their XPR1 receptor," *Retrovirology*, vol. 7, article 101, 2010.

[54] J. P. Stoye and J. M. Coffin, "Polymorphism of murine endogenous proviruses revealed by using virus class-specific oligonucleotide probes," *Journal of Virology*, vol. 62, no. 1, pp. 168–175, 1988.

[55] J. P. Stoye and J. M. Coffin, "The four classes of endogenous murine leukemia virus: structural relationships and potential for recombination," *Journal of Virology*, vol. 61, no. 9, pp. 2659–2669, 1987.

[56] S. C. Lo, N. Pripuzova, B. Li et al., "Detection of MLV-related virus gene sequences in blood of patients with chronic fatigue syndrome and healthy blood donors," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 36, pp. 15874–15879, 2010.

[57] Y. Yan, Q. Liu, K. Wollenberg, C. Martin, A. Buckler-White, and C. A. Kozak, "Evolution of functional and sequence variants of the mammalian XPR1 receptor for mouse xenotropic gammaretroviruses and the human-derived retrovirus XMRV," *Journal of Virology*, vol. 84, no. 22, pp. 11970–11980, 2010.

[58] Y. Yan, Q. Liu, and C. A. Kozak, "Six host range variants of the xenotropic/polytropic gammaretroviruses define determinants for entry in the XPR1 cell surface receptor," *Retrovirology*, vol. 6, article 87, 2009.

[59] P. Jern, G. O. Sperber, and J. Blomberg, "Divergent patterns of recent retroviral integrations in the human and chimpanzee genomes: probable transmissions between other primates and chimpanzees," *Journal of Virology*, vol. 80, no. 3, pp. 1367–1375, 2006.

[60] M. Marin, D. Lavillette, S. M. Kelly, and D. Kabat, "N-linked glycosylation and sequence changes in a critical negative control region of the ASCT1 and ASCT2 neutral amino acid transporters determine their retroviral receptor functions," *Journal of Virology*, vol. 77, no. 5, pp. 2936–2945, 2003.

[61] M. Marin, C. S. Tailor, A. Nouri, and D. Kabat, "Sodium-dependent neutral amino acid transporter type 1 is an auxiliary receptor for baboon endogenous retrovirus," *Journal of Virology*, vol. 74, no. 17, pp. 8085–8093, 2000.

[62] M. P. Kavanaugh and D. Kabat, "Identification and characterization of a widely expressed phosphate transporter/retrovirus receptor family," *Kidney International*, vol. 49, no. 4, pp. 959–963, 1996.

[63] M. P. Kavanaugh, H. Wang, C. A. Boyd, R. A. North, and D. Kabat, "Cell surface receptor for ecotropic host-range mouse retroviruses: a cationic amino acid transporter," *Archives of Virology*, vol. 9, supplement, pp. 485–494, 1994.

[64] J. L. Blond, D. Lavillette, V. Cheynet et al., "An envelope glycoprotein of the human endogenous retrovirus HERV-W is expressed in the human placenta and fuses cells expressing the type D mammalian retrovirus receptor," *Journal of Virology*, vol. 74, no. 7, pp. 3321–3329, 2000.

[65] Y. Yan, R. C. Knoper, and C. A. Kozak, "Wild mouse variants of envelope genes of xenotropic/polytropic mouse gammaretroviruses and their XPR1 receptors elucidate receptor determinants of virus entry," *Journal of Virology*, vol. 81, no. 19, pp. 10550–10557, 2007.

[66] E. C. Hawkins, "Saliva and tear tests for feline leukemia virus," *Journal of the American Veterinary Medical Association*, vol. 199, no. 10, pp. 1382–1385, 1991.

[67] O. Jarrett, "Overview of feline leukemia virus research," *Journal of the American Veterinary Medical Association*, vol. 199, no. 10, pp. 1279–1281, 1991.

[68] W. D. Hardy Jr., P. W. Hess, and M. Essex, "Horizontal transmission of feline leukemia virus in cats," *Bibliotheca Haematologica*, vol. no.40, pp. 67–74, 1975.

[69] J. Overbaugh, A. D. Miller, and M. V. Eiden, "Receptors and entry cofactors for retroviruses include single and multiple transmembrane-spanning proteins as well as newly described glycophosphatidylinositol-anchored and secreted proteins," *Microbiology and Molecular Biology Reviews*, vol. 65, no. 3, pp. 371–389, 2001.

[70] M. C. Adamson, J. Silver, and C. A. Kozak, "The mouse homolog of the Gibbon ape leukemia virus receptor: genetic mapping and a possible receptor function in rodents," *Virology*, vol. 183, no. 2, pp. 778–781, 1991.

[71] R. Mendoza, M. M. Anderson, and J. Overbaugh, "A putative thiamine transport protein is a receptor for feline leukemia virus subgroup A," *Journal of Virology*, vol. 80, no. 7, pp. 3378–3385, 2006.

[72] M. A. Sommerfelt and R. A. Weiss, "Receptor interference groups of 20 retroviruses plating on human cells," *Virology*, vol. 176, no. 1, pp. 58–69, 1990.

[73] A. C. van der Kuyl, J. T. Dekker, and J. Goudsmit, "Discovery of a new endogenous type C retrovirus (FcEV) in cats: evidence for RD-114 being an FcEV(Gag-Pol)/baboon endogenous virus BaEV(Env) recombinant," *Journal of Virology*, vol. 73, no. 10, pp. 7994–8002, 1999.

[74] Y. Takeuchi, F. L. C. Cosset, P. J. Lachmann, H. Okada, R. A. Weiss, and M. K. L. Collins, "Type C retrovirus inactivation by human complement is determined by both the viral genome and the producer cell," *Journal of Virology*, vol. 68, no. 12, pp. 8001–8007, 1994.

[75] H. C. T. Groom, M. W. Yap, R. P. Galão, S. J. D. Neil, and K. N. Bishop, "Susceptibility of xenotropic murine leukemia virus-related virus (XMRV) to retroviral restriction factors," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 11, pp. 5166–5171, 2010.

[76] K. Stieler and N. Fischer, "Apobec 3G efficiently reduces infectivity of the human exogenous gammaretrovirus XMRV," *PLoS ONE*, vol. 5, no. 7, Article ID e11738, 2010.

[77] L. Carthagena, M. C. Parise, M. Ringeard, M. K. Chelbi-Alix, U. Hazan, and S. Nisole, "Implication of TRIMalpha and TRIMCyp in interferon-induced anti-retroviral restriction activities," *Retrovirology*, vol. 5, article 59, 2008.

[78] B. Song, H. Javanbakht, M. Perron, D. H. Park, M. Stremlau, and J. Sodroski, "Retrovirus restriction by TRIM5α variants from Old World and New World primates," *Journal of Virology*, vol. 79, no. 7, pp. 3930–3937, 2005.

[79] M. W. Yap, S. Nisole, C. Lynch, and J. P. Stoye, "Trim5α protein restricts both HIV-1 and murine leukemia virus," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 29, pp. 10786–10791, 2004.

[80] C. M. Okeoma, N. Lovsin, B. M. Peterlin, and S. R. Ross, "APOBEC3 inhibits mouse mammary tumour virus replication in vivo," *Nature*, vol. 445, no. 7130, pp. 927–930, 2007.

[81] E. Takeda, S. Tsuji-Kawahara, M. Sakamoto et al., "Mouse APOBEC3 restricts friend leukemia virus infection and pathogenesis in vivo," *Journal of Virology*, vol. 82, no. 22, pp. 10998–11008, 2008.

[82] E. C. Knouf, M. J. Metzger, P. S. Mitchell et al., "Multiple integrated copies and high-level production of the human retrovirus XMRV (xenotropic murine leukemia virus-related virus) from 22Rv1 prostate carcinoma cells," *Journal of Virology*, vol. 83, no. 14, pp. 7353–7356, 2009.

[83] K. Stieler, C. Schulz, M. Lavanya, M. Aepfelbacher, C. Stocking, and N. Fischer, "Host range and cellular tropism of the human exogenous gammaretrovirus XMRV," *Virology*, vol. 399, no. 1, pp. 23–30, 2010.

[84] O. Hohn, K. Strohschein, A. U. Brandt et al., "No evidence for XMRV in German CFS and MS patients with fatigue despite the ability of the virus to infect human blood cells in vitro," *PLoS ONE*, vol. 5, no. 12, Article ID e15632, 2010.

[85] M. Cornelissen, F. Zorgdrager, P. Blom et al., "Lack of detection of XMRV in seminal plasma from HIV-1 infected men in The Netherlands," *PLoS ONE*, vol. 5, no. 8, Article ID e12040, 2010.

[86] E. Barnes, P. Flanagan, A. Brown et al., "Failure to detect xenotropic murine leukemia virus-related virus in blood of individuals at high risk of blood-borne viral infections," *Journal of Infectious Diseases*, vol. 202, no. 10, pp. 1482–1485, 2010.

[87] B. P. Danielson, G. E. Ayala, and J. T. Kimata, "Detection of xenotropic murine leukemia virus-related virus in normal and tumor tissue of patients from the southern United States with prostate cancer is dependent on specific polymerase chain reaction conditions," *Journal of Infectious Diseases*, vol. 202, no. 10, pp. 1470–1477, 2010.

[88] N. Bannert, "Editorial: is a novel human retrovirus associated with prostate cancer and chronic fatigue syndrome?" *Future Microbiology*, vol. 5, no. 5, pp. 689–691, 2010.

[89] O. Erlwein, S. Kaye, M. O. McClure et al., "Failure to detect the novel retrovirus XMRV in chronic fatigue syndrome," *PLoS ONE*, vol. 5, no. 1, Article ID e8519, 2010.

[90] H. C. T. Groom, V. C. Boucherit, K. Makinson et al., "Absence of xenotropic murine leukaemia virus-related virus in UK patients with chronic fatigue syndrome," *Retrovirology*, vol. 7, article 10, 2010.

[91] T. J. Henrich, J. Z. Li, D. Felsenstein et al., "Xenotropic murine leukemia virus-related virus prevalence in patients with chronic fatigue syndrome or chronic immunomodulatory conditions," *Journal of Infectious Diseases*, vol. 202, no. 10, pp. 1478–1481, 2010.

[92] P. Hong, J. Li, and Y. Li, "Failure to detect Xenotropic murine leukaemia virus-related virus in Chinese patients with chronic fatigue syndrome," *Virology Journal*, vol. 7, article 224, 2010.

[93] S. Hue, E. R. Gray, A. Gall et al., "Disease-associated XMRV sequences are consistent with laboratory contamination," *Retrovirology*, vol. 7, article 111, 2010.

[94] B. Oakes, A. K. Tai, O. Cingoz et al., "Contamination of human DNA samples with mouse DNA can lead to false detection of XMRV-like sequences," *Retrovirology*, vol. 7, article 109, 2010.

[95] M. J. Robinson, O. W. Erlwein, S. Kaye et al., "Mouse DNA contamination in human tissue tested for XMRV," *Retrovirology*, vol. 7, article 108, 2010.

[96] J. Kaiser, "Chronic fatigue syndrome. Studies point to possible contamination in XMRV findings," *Science*, vol. 331, no. 6013, p. 17, 2011.

[97] G. W. Verhaegh, A. S. de Jong, F. P. Smit, S. A. Jannink, W. J.G. Melchers, and J. A. Schalken, "Prevalence of human xenotropic murine leukemia virus-related gammaretrovirus (XMRV) in dutch prostate cancer patients," *Prostate*, vol. 71, no. 4, pp. 415–420, 2011.

[98] F. J. van Kuppeveld, A. S. de Jong, K. H. Lanke et al., "Prevalence of xenotropic murine leukaemia virus-related virus in patients with chronic fatigue syndrome in the Netherlands: retrospective analysis of samples from an established cohort," *British Medical Journal*, vol. 340, article c1018, 2010.

[99] J. A. Garson, P. Kellam, and G. J. Towers, "Analysis of XMRV integration sites from human prostate cancer tissues suggests PCR contamination rather than genuine human infection," *Retrovirology*, vol. 8, article 13, 2011.

[100] R. A. Smith, "Contamination of clinical specimens with MLV-encoding nucleic acids: implications for XMRV and other candidate human retroviruses," *Retrovirology*, vol. 7, article 112, 2010.

[101] E. Sato, R. A. Furuta, and T. Miyazawa, "An endogenous murine leukemia viral genome contaminant in a commercial RT-PCR Kit is amplified using standard primers for XMRV," *Retrovirology*, vol. 7, article 110, 2010.

[102] J. A. Mikovits, Y. Huang, M. A. Pfost et al., "Distribution of xenotropic murine leukemia virus-related virus (XMRV) infection in chronic fatigue syndrome and prostate cancer," *AIDS Reviews*, vol. 12, no. 3, pp. 149–152, 2010.

[103] J. A. Mikovits, V. C. Lombardi, M. A. Pfost, K. S. Hagen, and F. W. Ruscetti, "Detection of an infectious retrovirus, XMRV, in blood cells of patients with chronic fatigue syndrome," *Virulence*, vol. 1, no. 5, pp. 386–390, 2010.

[104] R. S. Arnold, N. V. Makarova, A. O. Osunkoya et al., "XMRV infection in patients with prostate cancer: novel serologic assay and correlation with PCR and FISH," *Urology*, vol. 75, no. 4, pp. 755–761, 2010.

[105] W. M. Switzer, H. Jia, O. Hohn et al., "Absence of evidence of xenotropic murine leukemia virus-related virus infection in persons with chronic fatigue syndrome and healthy controls in the United States," *Retrovirology*, vol. 7, article 57, 2010.

[106] O. Hohn, H. Krause, P. Barbarotto et al., "Lack of evidence for xenotropic murine leukemia virus-related virus(XMRV) in German prostate cancer patients," *Retrovirology*, vol. 6, article 92, 2009.

[107] S. Sabunciyan, N. Mandelberg, C. S. Rabkin, R. Yolken, and R. Viscidi, "No difference in antibody titers against xenotropic MLV related virus in prostate cancer cases and cancer-free controls," *Molecular and Cellular Probes*, vol. 25, no. 2-3, pp. 134–136, 2011.

[108] R. A. Furuta, T. Miyazawa, T. Sugiyama et al., "No association of xenotropic murine leukemia virus-related virus with prostate cancer or chronic fatigue syndrome in Japan," *Retrovirology*, vol. 8, article 20, 2011.

[109] X. Qiu, P. Swanson, K. C. Luk et al., "Characterization of antibodies elicited by XMRV infection and development of immunoassays useful for epidemiologic studies," *Retrovirology*, vol. 7, article 68, 2010.

[110] R. Schlaberg, D. J. Choe, K. R. Brown, H. M. Thaker, and I. R. Singh, "XMRV is present in malignant prostatic epithelium and is associated with prostate cancer, especially high-grade tumors," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 38, pp. 16351–16356, 2009.

[111] C. Y. Chiu, S. Rouskin, A. Koshy et al., "Microarray detection of human parainfluenzavirus 4 infection associated with respiratory failure in an immunocompetent adult," *Clinical Infectious Diseases*, vol. 43, no. 8, pp. e71–e76, 2006.

[112] V. R. Placencio, A. R. Sharif-Afshar, X. Li et al., "Stromal transforming growth factor-β signaling mediates prostatic response to androgen ablation by paracrine Wnt activity," *Cancer Research*, vol. 68, no. 12, pp. 4709–4718, 2008.

[113] N. A. Bhowmick, E. G. Neilson, and H. L. Moses, "Stromal fibroblasts in cancer initiation and progression," *Nature*, vol. 432, no. 7015, pp. 332–337, 2004.

[114] V. Montel, E. S. Mose, and D. Tarin, "Tumor-stromal interactions reciprocally modulate gene expression patterns during carcinogenesis and metastasis," *International Journal of Cancer*, vol. 119, no. 2, pp. 251–263, 2006.

[115] D. Tarin, "Tissue interactions in morphogenesis, morphostasis and carcinogenesis," *Journal of Theoretical Biology*, vol. 34, no. 1, pp. 61–72, 1972.

[116] A. F. Olumi, G. D. Grossfeld, S. W. Hayward, P. R. Carroll, T. D. Tlsty, and G. R. Cunha, "Carcinoma-associated fibroblasts direct tumor progression of initiated human prostatic epithelium," *Cancer Research*, vol. 59, no. 19, pp. 5002–5011, 1999.

[117] S. W. Hayward, Y. Wang, M. Cao et al., "Malignant transformation in a nontumorigenic human prostatic epithelial cell line," *Cancer Research*, vol. 61, no. 22, pp. 8135–8142, 2001.

[118] L. A. Jason, A. Boulton, N. S. Porter, T. Jessen, M. G. Njoku, and F. Friedberg, "Classification of myalgic encephalomyelitis/chronic fatigue syndrome by types of fatigue," *Behavioral Medicine*, vol. 36, no. 1, pp. 24–31, 2010.

[119] O. Zachrisson, B. Regland, M. Jahreskog, M. Kron, and C. G. Gottfries, "A rating scale for fibromyalgia and chronic fatigue syndrome (the FibroFatigue scale)," *Journal of Psychosomatic Research*, vol. 52, no. 6, pp. 501–509, 2002.

[120] J. Blomberg, "Swedish study comes up empty," in *Proceedings of the 1st XMRV Conference*, Bethesda, Md, USA, 2010.

[121] L. Zhang, J. Gough, D. Christmas et al., "Microbial infections in eight genomic subtypes of chronic fatigue syndrome/myalgic encephalomyelitis," *Journal of Clinical Pathology*, vol. 63, no. 2, pp. 156–164, 2010.

[122] K. Mørch, K. Hanevik, G. Rortveit et al., "Severity of Giardia infection associated with post-infectious fatigue and abdominal symptoms two years after," *BMC Infectious Diseases*, vol. 9, article 206, 2009.

[123] K. Mørch, K. Hanevik, G. Rortveit, K. A. Wensaas, and N. Langeland, "High rate of fatigue and abdominal symptoms 2 years after an outbreak of giardiasis," *Transactions of the Royal Society of Tropical Medicine and Hygiene*, vol. 103, no. 5, pp. 530–532, 2009.

[124] M. B. Gardner, C. A. Kozak, and S. J. O'Brien, "The Lake Casitas wild mouse: evolving genetic resistance to retroviral disease," *Trends in Genetics*, vol. 7, no. 1, pp. 22–27, 1991.

[125] O. Jarrett, "Pathogenicity of feline leukemia virus is commonly associated with variant viruses," *Leukemia*, vol. 6, supplement 3, pp. 153S–154S, 1992.

[126] R. R. Watson, "Resistance to intestinal parasites during murine AIDS: role of alcohol and nutrition in immune dysfunction," *Parasitology*, vol. 107, supplement, pp. S69–S74, 1993.

[127] P. Jolicoeur, "Murine acquired immunodeficiency syndrome (MAIDS): an animal model to study the AIDS pathogenesis," *FASEB Journal*, vol. 5, no. 10, pp. 2398–2405, 1991.

[128] C. S. Via, H. C. Morse III, and G. M. Shearer, "Altered immunoregulation and autoimmune aspects of HIV infection: relevant murine models," *Immunology Today*, vol. 11, no. 7, pp. 250–255, 1990.

[129] R. R. Watson, "A murine retroviurs model for studies of the role of cofactors and ethanol in AIDS development," *NIDA Research Monograph Series*, no. 96, pp. 166–180, 1990.

[130] R. R. Watson, "Murine models for acquired immune deficiency syndrome," *Life Sciences*, vol. 44, no. 3, pp. 3–15, 1989.

[131] E. A. Hoover, S. L. Quackenbush, M. L. Poss et al., "Pathogenic mechanisms of immunodeficiency syndrome and aplastic anemia induced by feline leukemia viruses," *Developments in Biological Standardization*, vol. 72, pp. 163–166, 1990.

[132] J. Overbaugh, P. R. Donahue, S. L. Quackenbush, E. A. Hoover, and J. I. Mullins, "Molecular cloning of a feline leukemia virus that induces fatal immunodeficiency disease in cats," *Science*, vol. 239, no. 4842, pp. 906–910, 1988.

[133] N. Tabata, M. Miyazawa, R. Fujisawa, Y. A. Takei, H. Abe, and K. Hashimoto, "Establishment of monoclonal antiretroviral gp70 autoantibodies from MRL/lpr lupus mice and

induction of glomerular gp70 deposition and pathology by transfer into non-autoimmune mice," *Journal of Virology*, vol. 74, no. 9, pp. 4116–4126, 2000.

[134] S. K. Datta, P. J. McConahey, N. Manny, A. N. Theofilopoulos, F. J. Dixon, and R. S. Schwartz, "Genetic studies of autoimmunity and retrovirus expression in crosses of New Zealand Black mice. II. The viral envelope glycoprotein gp70," *Journal of Experimental Medicine*, vol. 147, no. 3, pp. 872–881, 1978.

[135] R. Schlaberg, D. J. Choe, K. R. Brown, H. M. Thaker, and I. R. Singh, "XMRV is present in malignant prostatic epithelium and is associated with prostate cancer, especially high-grade tumors," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 38, pp. 16351–16356, 2009.

[136] Y. Takeuchi, M. O. McClure, and M. Pizzato, "Identification of gammaretroviruses constitutively released from cell lines used for human immunodeficiency virus research," *Journal of Virology*, vol. 82, no. 24, pp. 12585–12588, 2008.

[137] M. Dewannieux, D. Ribet, and T. Heidmann, "Risks linked to endogenous retroviruses for vaccine production: a general overview," *Biologicals*, vol. 38, no. 3, pp. 366–370, 2010.

[138] R. A. Weiss, "A cautionary tale of virus and disease," *BMC Biology*, vol. 8, article 124, 2010.

[139] C. Voisset, R. A. Weiss, and D. J. Griffiths, "Human RNA "rumor" viruses: the search for novel human retroviruses in chronic disease," *Microbiology and Molecular Biology Reviews*, vol. 72, no. 1, pp. 157–196, 2008.

[140] A. Forsman, D. Uzameckis, L. Rönnblom et al., "Single-tube nested quantitative PCR: a rational and sensitive technique for detection of retroviral DNA. Application to RERV-H/HRV-5 and confirmation of its rabbit origin," *Journal of Virological Methods*, vol. 111, no. 1, pp. 1–11, 2003.

[141] J. W. Hartley, L. H. Evans, K. Y. Green et al., "Expression of infectious murine leukemia viruses by RAW264.7 cells, a potential complication for studies with a widely used mouse macrophage cell line," *Retrovirology*, vol. 5, article 1, 2008.

[142] A. Stang, E. Petrasch-Parwez, S. Brandt et al., "Unintended spread of a biosafety level 2 recombinant retrovirus," *Retrovirology*, vol. 6, article 86, 2009.

[143] R. Yoshikawa, E. Sato, and T. Miyazawa, "Contamination of infectious RD-114 virus in vaccines produced using non-feline cell lines," *Biologicals*, vol. 39, no. 1, pp. 33–37, 2011.

[144] T. F. Malivanova and S. V. Litvinov, "Antibodies to retroviruses of types C and D in female patients with benign and malignant mammary tumours," *Acta Virologica*, vol. 34, no. 1, pp. 19–26, 1990.

[145] R. H. Reeves and S. J. O'Brien, "Molecular genetic characterization of the RD-114 gene family of endogenous feline retroviral sequences," *Journal of Virology*, vol. 52, no. 1, pp. 164–171, 1984.

[146] P. Herbrink, J. E. T. Moen, J. Brouwer, and S. O. Warnaar, "Detection of antibodies cross-reactive with type C RNA tumor viral p30 protein in human sera and exudate fluids," *Cancer Research*, vol. 40, no. 1, pp. 166–173, 1980.

[147] K. Welte, U. Ebener, and P. Chandra, "Serological characterization of a purified reverse transcriptase from osteosarcoma of a child," *Cancer Letters*, vol. 7, no. 4, pp. 189–195, 1979.

[148] P. Chandra, S. Balikcioglu, and B. Mildner, "Biochemical and immunological characterization of a reverse transcriptase from human melanoma tissue," *Cancer Letters*, vol. 5, no. 6, pp. 299–310, 1978.

[149] H. S. Kaplan, R. S. Goodenow, A. L. Epstein, S. Gartner, A. Decleve, and P. N. Rosenthal, "Isolation of a type C RNA virus from an established human histiocytic lymphoma cell line," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 74, no. 6, pp. 2564–2568, 1977.

[150] M. Strand and J. T. August, "Type C RNA virus gene expression in human tissue," *Journal of Virology*, vol. 14, no. 6, pp. 1584–1596, 1974.

[151] R. M. McAllister, M. Nicolson, and M. B. Gardner, "RD 114 virus compared with feline and murine type C viruses released from RD cells," *Nature*, vol. 242, no. 116, pp. 75–78, 1973.

[152] K. V. Ilyin, J. G. Kzhyshkowska, L. R. Imamova et al., "Type D retrovirus specific sequences in lymphocytes of the children with Burkitt-type lymphoma and their parents," *Immunology Letters*, vol. 78, no. 1, pp. 51–54, 2001.

[153] W. Hofmann, B. Reichart, A. Ewald et al., "Cofactor requirements for nuclear export of Rev response element (RRE)- and constitutive transport element (CTE)-containing retroviral RNAs: an unexpected role for actin," *Journal of Cell Biology*, vol. 152, no. 5, pp. 895–910, 2001.

[154] J. G. Kzhyshkowska, A. V. Kiselev, G. A. Gordina et al., "Markers of type D retroviruses in children with Burkitt's-type lymphoma," *Immunology Letters*, vol. 53, no. 2-3, pp. 101–104, 1996.

[155] R. J. Ford, L. A. Donehower, and R. C. Bohannon, "Studies on a type D retrovirus isolated from an AIDS patient lymphoma," *AIDS Research and Human Retroviruses*, vol. 8, no. 5, pp. 742–751, 1992.

[156] R. C. Bohannon, L. A. Donehower, and R. J. Ford, "Isolation of a type D retrovirus from B-cell lymphomas of a patient with AIDS," *Journal of Virology*, vol. 65, no. 11, pp. 5663–5672, 1991.

[157] L. A. Donehower, R. C. Bohannon, R. J. Ford, and R. A. Gibbs, "The use of primers from highly conserved pol regions to identify uncharacterized retorviruses by the polymerase chain reaction," *Journal of Virological Methods*, vol. 28, no. 1, pp. 33–46, 1990.

[158] H. Krause, W. Uckert, and V. Wunderlich, "Use of molecular cloned viral genomes for studies into interrelationship between type D retroviruses from monkey and human cells," *Archiv fur Experimentelle Veterinarmedizin*, vol. 44, no. 6, pp. 931–933, 1990.

[159] H. Krause, V. Wunderlich, and W. Uckert, "Molecular cloning of a type D retrovirus from human cells (PMFV) and its homology to simian acquired immunodeficiency type D retroviruses," *Virology*, vol. 173, no. 1, pp. 214–222, 1989.

[160] D. F. J. Purcell, N. J. Deacon, and I. F. C. McKenzie, "The human non-lineage antigen CD46 (HuLy-m5) and primate retroviral gp70 molecules share protein-defined antigenic determinants," *Immunology and Cell Biology*, vol. 67, no. 5, pp. 279–289, 1989.

[161] M. Popovic, V. S. Kalyanaraman, M. S. Reitz, and M. G. Sarngadharan, "Identification of the RPMI 8226 retrovirus and its dissemination as a significant contaminant of some widely used human and marmoset cell lines," *International Journal of Cancer*, vol. 30, no. 1, pp. 93–99, 1982.

[162] M. Fukuda, H. J. Wanebo, and L. Tsuei, "Leukocyte migration inhibition among breast cancer patients in response to various oncogenic viruses," *Journal of the National Cancer Institute*, vol. 64, no. 3, pp. 431–437, 1980.

[163] W. Uckert, V. Wunderlich, and E. Bender, "The protein pattern of PMF virus, a type-D retrovirus from malignant

permanent human cell lines," *Archives of Virology*, vol. 64, no. 2, pp. 155–166, 1980.

[164] L. Thiry, S. Sprecher-Goldberger, M. Bossens, and F. Neuray, "Cell mediated immune response to simian oncornavirus antigens in pregnant women," *Journal of the National Cancer Institute*, vol. 60, no. 3, pp. 527–532, 1978.

[165] S. G. Devare, L. O. Arthur, D. L. Fine, and J. R. Stephenson, "Primate retroviruses: immunological cross-reactivity between major structural proteins of new and old world primate virus isolates," *Journal of Virology*, vol. 25, no. 3, pp. 797–805, 1978.

[166] A. Malmsten, D. H. L. Ekstrand, L. Åkerblom et al., "A colorimetric reverse transcriptase assay optimized for Moloney murine leukemia virus, and its use for characterization of reverse transcriptases of unknown identity," *Journal of Virological Methods*, vol. 75, no. 1, pp. 9–20, 1998.

[167] J. F. Sears, R. Repaske, and A. S. Khan, "Improved Mg^{2+}-based reverse transcriptase assay for detection of primate retroviruses," *Journal of Clinical Microbiology*, vol. 37, no. 6, pp. 1704–1708, 1999.

[168] H. Pyra, J. Boni, and J. Schupbach, "Ultrasensitive retrovirus detection by a reverse transcriptase assay based on product enhancement," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 91, no. 4, pp. 1544–1548, 1994.

[169] N. Fischer, C. Schulz, K. Stieler et al., "Xenotropic murine leukemia virus-related gammaretro virus in respiratory tract," *Emerging Infectious Diseases*, vol. 16, no. 6, pp. 1000–1002, 2010.

[170] K. Lee, F. W. Ruscetti, P. Lloyd, A. Rein, G. Fanning-Heidecker, and V. N. Kewalramani, "Development of a GFP-indicator cell line for the detection of XMRV," in *Proceedings of the 1st XMRV conference*, National Institutes of Health, Bethesda, Md, USA, 2010.

[171] M. Deichmann, J. B. Huder, C. Kleist, H. Näher, J. Schüpbach, and J. Böni, "Detection of reverse transcriptase activity in human melanoma cell lines and identification of a murine leukemia virus contaminant," *Archives of Dermatological Research*, vol. 296, no. 8, pp. 345–352, 2005.

[172] R. Yoshikawa, E. Sato, T. Igarashi, and T. Miyazawa, "Characterization of RD-114 virus isolated from a commercial canine vaccine manufactured using CRFK cells," *Journal of Clinical Microbiology*, vol. 48, no. 9, pp. 3366–3369, 2010.

[173] T. Miyazawa, "Endogenous retroviruses as potential hazards for vaccines," *Biologicals*, vol. 38, no. 3, pp. 371–376, 2010.

[174] T. Miyazawa, R. Yoshikawa, M. Golder, M. Okada, H. Stewart, and M. Palmarini, "Isolation of an infectious endogenous retrovirus in a proportion of live attenuated vaccines for pets," *Journal of Virology*, vol. 84, no. 7, pp. 3690–3694, 2010.

[175] M. Zhang, S. Lute, L. Norling et al., "A novel, Q-PCR based approach to measuring endogenous retroviral clearance by capture protein a chromatography," *Biotechnology and Bioengineering*, vol. 102, no. 5, pp. 1438–1447, 2009.

[176] A. Chang and S. Dusing, "Practical assay issues with the PERT/PBRT assay: a highly sensitive reverse transcriptase assay," *Developments in Biologicals*, vol. 123, pp. 91–97, 2006.

[177] J. Blomberg, G. Folsch, I. Nilsson, and R. Faldt, "Immunoglobulin G antibodies binding to a synthetic peptide deduced from the nucleotide sequence of the env gene of HTLV I in patients with leukemia and rheumatoid arthritis, HLA sensitized persons and blood donors," *Leukemia Research*, vol. 9, no. 9, pp. 1111–1116, 1985.

[178] J. Blomberg, A. Lawoko, R. Pipkorn et al., "A survey of synthetic HIV-1 peptides with natural and chimeric sequences for differential reactivity with Zimbabwean, Tanzanian and Swedish HlV-1-positive sera," *AIDS*, vol. 7, no. 6, pp. 759–767, 1993.

[179] J. Blomberg, T. Moestrup, J. Frimand et al., "HTLV-I and -II in intravenous drug users from Sweden and Denmark," *Scandinavian Journal of Infectious Diseases*, vol. 26, no. 1, pp. 23–26, 1994.

[180] J. Blomberg, M. Robert-Guroff, W. A. Blattner, and R. Pipkorn, "Type- and group-specific continuous antigenic determinants of HTLV. Use of synthetic peptides for serotyping of HTLV-I and -II infection," *Journal of Acquired Immune Deficiency Syndromes*, vol. 5, no. 3, pp. 294–302, 1992.

[181] J. Blomberg, E. Vincic, C. Jonsson, P. Medstrand, and R. Pipkorn, "Identification of regions of HIV-1 p24 reactive with sera which give "indeterminate" results in electrophoretic immunoblots with the help of long synthetic peptides," *AIDS Research and Human Retroviruses*, vol. 6, no. 12, pp. 1363–1372, 1990.

[182] P. J. Klasse, R. Pipkorn, and J. Blomberg, "Presence of antibodies to a putatively immunosuppressive part of human immunodeficiency virus (HIV) envelope glycoprotein gp41 is strongly associated with health among HIV-positive subjects," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 85, no. 14, pp. 5225–5229, 1988.

[183] N. Onlamoon, J. D. Gupta, P. Sharma et al., "Infection, viral dissemination, and antibody responses of rhesus macaques exposed to the human gammaretrovirus XMRV," *Journal of Virology*, vol. 85, no. 9, pp. 4547–4557, 2011.

[184] T. Sakuma, J. M. Tonne, K. A. Squillace et al., "Early events in retrovirus XMRV infection of the wild-derived mouse Mus pahari," *Journal of Virology*, vol. 85, no. 3, pp. 1205–1213, 2011.

[185] W. K. Gillette, D. Esposito, T. E. Taylor, R. F. Hopkins, R. K. Bagni, and J. L. Hartley, "Purify First: rapid expression and purification of proteins from XMRV," *Protein Expression and Purification*, vol. 76, no. 2, pp. 238–247, 2011.

[186] E. P. Lillehoj, G. M. Ford, S. Bachmann, J. Schröder, E. F. Torrey, and R. H. Yolken, "Serum antibodies reactive with non-human primate retroviruses identified in acute onset schizophrenia," *Journal of NeuroVirology*, vol. 6, no. 6, pp. 492–497, 2000.

[187] D. K. Langat, E. O. Wango, G. O. Owiti, E. O. Omollo, and J. M. Mwenda, "Characterisation of retroviral-related antigens expressed in normal baboon placental tissues," *African Journal of Health Sciences*, vol. 5, pp. 144–152, 1998.

[188] B. Szabo, F. D. Toth, J. Kiss, A. Kiss, and K. Rak, "Antiretroviral immune response and plasma interferon in different phases of chronic granulocytic leukemia," *Leukemia Research*, vol. 17, no. 4, pp. 311–323, 1993.

[189] C. Leib-Mosch, M. Bachmann, R. Brack-Werner, T. Werner, V. Erfle, and R. Hehlmann, "Expression and biological significance of human endogenous retroviral sequences," *Leukemia*, vol. 6, supplement 3, pp. 72S–75S, 1992.

[190] J. Kiss, F. D. Toth, B. Szabo, A. Kiss, and K. Rak, "Retrovirus-neutralizing antibodies in AML and AMMoL patients: stage-specific distribution," *Neoplasma*, vol. 36, no. 2, pp. 191–197, 1989.

[191] F. D. Tóth, L. Váczi, B. Szabó et al., "Detection of main core proteins of simian C-type viruses and human retrovirus HTLV and antibodies to them in patients with lymphoid malignancies," *Acta Microbiologica Hungarica*, vol. 32, no. 3, pp. 267–273, 1985.

[192] S. Maeda, K. Yonezawa, and A. Yachi, "Serum antibody reacting with placental syncytiotrophoblast in sera of patients

with autoimmune diseases—a possible relation to type C RNA retrovirus," *Clinical and Experimental Immunology*, vol. 60, no. 3, pp. 645–653, 1985.

[193] F. D. Toth, L. Vaczi, B. Szabo et al., "Studies on the specificity of human antibodies reacting with GP70 and P15 antigens of baboon endogenous (BaEV) and gibbon ape leukaemia (GaLV) viruses," *Acta Virologica*, vol. 28, no. 3, pp. 191–197, 1984.

[194] B. Szabó, F. D. Tóth, L. Váczi, A. Réthy, A. Kiss, and K. Rák, "Cellular and humoral cytotoxicity in patients with leukaemia and preleukaemia. II. Oncovirus specificity of the reaction," *Acta Microbiologica Hungarica*, vol. 31, no. 4, pp. 365–372, 1984.

[195] N. Anh-Tuan, F. D. Toth, B. Szabo et al., "Antibodies to primate retrovirus antigens in circulating immune complexes of patients with acute myeloid leukemia," *Leukemia Research*, vol. 8, no. 5, pp. 863–871, 1984.

[196] S. Maeda, R. C. Mellors, and J. W. Mellors, "Immunohistologic detection of antigen related to primate type C retrovirus p30 in normal human placentas," *American Journal of Pathology*, vol. 112, no. 3, pp. 347–356, 1983.

[197] R. Hehlmann, H. Schetters, V. Erfle, and C. Leib-Mosch, "Detection and biochemical characterization of antigens in human leukemic sera that cross-react with primate C-type viral proteins (M(r) 30,000)," *Cancer Research*, vol. 43, no. 1, pp. 392–399, 1983.

[198] D. Wernicke and R. Kurth, "Human antibodies recognizing the envelope glycoprotein of the baboon endogenous virus BaEV are of heterophil origin," *Medical Microbiology and Immunology*, vol. 170, no. 2, pp. 135–143, 1981.

[199] F. D. Toth, L. Vaczi, and J. Kiss, "Detection by radioimmunoassay of antigens related to the P30 polypeptides of primate type C oncoviruses in peripheral leukocytes from patients with chronic myelogenous leukaemia," *Acta Virologica*, vol. 25, no. 6, pp. 376–380, 1981.

[200] L. E. Posner, M. Robert-Guroff, and V. S. Kalyanaraan, "Natural antibodies to the human T cell lymphoma virus in patients with cutaneous T cell lymphomas," *Journal of Experimental Medicine*, vol. 154, no. 2, pp. 333–346, 1981.

[201] S. A. Stass, T. M. Phillips, O. S. Weislow, E. Perlin, and H. R. Schumacher, "Antigen-antibody complexes related to the baboon endogenous virus in humans with acute lymphoblastic leukemia-hand mirror variant (ALL-HMC)," *Blood*, vol. 56, no. 4, pp. 661–666, 1980.

[202] F. Wong-Staal, S. Josephs, R. D. Favera, and R. Gallo, "Detection of integrated type-C viral DNA fragments in two primates (human and gibbon) by the restriction enzyme blotting technique," *Hamatologie und Bluttransfusion*, vol. 23, pp. 553–560, 1979.

[203] J. Blomberg, O. Nived, R. Pipkorn, A. Bengtsson, D. Erlinge, and G. Sturfelt, "Increased antiretroviral antibody reactivity in sera from a defined population of patients with systemic lupus erythematosus: correlation with autoantibodies and clinical manifestations," *Arthritis and Rheumatism*, vol. 37, no. 1, pp. 57–66, 1994.

[204] B. C. Satterfield, R. A. Garcia, H. Jia, S. Tang, H. Zheng, and W. M. Switzer, "Serologic and PCR testing of persons with chronic fatigue syndrome in the United States shows no association with xenotropic or polytropic murine leukemia virus-related viruses," *Retrovirology*, vol. 8, article 12, 2011.

[205] M. J. Metzger and A. D. Miller, "Acutely transforming retrovirus expressing Nras generated from HT-1080 fibrosarcoma cells infected with the human retrovirus XMRV," *Journal of Virology*, vol. 84, no. 15, pp. 7908–7910, 2010.

[206] A. Lawoko, B. Johansson, D. Rabinayaran, R. Pipkorn, and J. Blomberg, "Increased immunoglobulin G, but not M, binding to endogenous retroviral antigens in HIV-1 infected persons," *Journal of Medical Virology*, vol. 62, no. 4, pp. 435–444, 2000.

[207] W. J. Martin, "Possible widespread low-level occurrence of murine leukemia virus-related gene sequences in humans," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 43, p. E162, 2010.

[208] A. Krook and J. Blomberg, "HTLV-II among injecting drug users in Stockholm," *Scandinavian Journal of Infectious Diseases*, vol. 26, no. 2, pp. 129–132, 1994.

[209] A. Krook, J. Albert, S. Andersson et al., "Prevalence and risk factors for HTLV-II infection in 913 injecting drug users in Stockholm, 1994," *Journal of Acquired Immune Deficiency Syndromes and Human Retrovirology*, vol. 15, no. 5, pp. 381–386, 1997.

[210] M. Zanchetta, A. Anselmi, D. Vendrame et al., "Early therapy in HIV-1-infected children: effect on HIV-1 dynamics and HIV-1-specific immune response," *Antiviral Therapy*, vol. 13, no. 1, pp. 47–55, 2008.

[211] C. Pastori, C. Barassi, F. Lillo et al., "The effect of HAART on humoral immune response in primary HIV-1 infected patients," *Journal of Biological Regulators and Homeostatic Agents*, vol. 16, no. 1, pp. 9–17, 2002.

[212] H. G. Klein, R. Y. Dodd, F. B. Hollinger et al., "Xenotropic murine leukemia virus-related virus (XMRV) and blood transfusion: report of the AABB interorganizational XMRV task force," *Transfusion*, vol. 51, no. 3, pp. 654–661, 2011.

[213] R. A. Smith, G. S. Gottlieb, and A. D. Miller, "Susceptibility of the human retrovirus XMRV to antiretroviral inhibitors," *Retrovirology*, vol. 7, article 70, 2010.

Hindawi Publishing Corporation
Advances in Virology
Volume 2011, Article ID 813651, 16 pages
doi:10.1155/2011/813651

Review Article

MoMuLV-ts-1: A Unique Mouse Model of Retrovirus-Induced Lymphoma Transmitted by Breast Milk

J. Chakraborty,[1] H. Okonta,[1,2] H. Bagalb,[1] and J. Duggan[1,2]

[1] *Department of Physiology and Pharmacology, Block Health Science Building, College of Medicine, University of Toledo, Health Science Campus, Toledo, OH 43614, USA*
[2] *Department of Medicine, Ruppert Building, College of Medicine, University of Toledo, Health Science Campus, Toledo, OH 43614, USA*

Correspondence should be addressed to J. Chakraborty, joana.chakraborty@utoledo.edu

Received 31 January 2011; Revised 16 May 2011; Accepted 6 June 2011

Academic Editor: Paul Jolicoeur

Our laboratory has developed a murine model of lymphoma via breast milk transmission of MoMuLV-ts-1 (Moloney murine leukemia virus-temperature sensitive mutant-1). Uninfected offspring suckled from infected surrogate mothers become infected and develop lymphoma. Multiple gene integration sites of ts-1 into the infected mouse genome including *tacc3, aurka, ndel1, tpx2, p53, and rhamm* were identified, and mRNA expressions were quantitated. These genes produce centrosomal proteins, which may be involved in abnormal chromosomal segregation leading to aneuploidy or multiploidy, thus causing lymphoma. Since there is no report to date on this retroviral model leading to centrosomal abnormality, and causing lymphoma development, this is a valuable and unique model to study the centrosomal involvement in lymphomagenesis.

1. Introduction

Retroviral infections in humans range from asymptomatic murine leukemia virus infections to deadly infections such as HIV. The HIV epidemic has spurred unprecedented levels of retroviral research in the last three decades due to the ever-increasing human toll. According to the WHO and UNAIDS report in 2008 [1], globally 33.4 million people are now living with HIV. Of these, 2.1 million children under the age of 15 years are suffering from HIV infection and 280,000 children died of AIDS and AIDS-related conditions such as lymphoma in 2008. In 2008, an additional 430,000 children became infected, with the vast majority living in developing countries with little access to antiretroviral therapy (ART). The main route of infection in these children is mother to child transmission (MTCT). Although treatment of infected mothers during pregnancy with ART and use of infant formula after delivery has limited MTCT of HIV-1 in developed countries [1], these options are often unavailable in Africa and other developing countries where MTCT still remains a significant source of HIV infection.

Due to practical and ethical constraints involving human subjects, the mechanism of perinatal transmission of HIV-1 is not yet fully understood. HIV can be transmitted to the fetus/infant during prenatal and postnatal periods. A suitable animal model may allow in depth study of the pathophysiologic mechanisms of MTCT of HIV. We have successfully developed a murine model for mother-to-offspring transmission of Moloney Murine Leukemia virus (MoMuLV) ts-1, a retrovirus which mimics HIV. We have clearly demonstrated that the transmission of ts-1 can occur in utero, intrapartum, and/or postpartum. Transmission of ts-1 produces an immunodeficiency state with wasting, increased infection, and neurologic deterioration. In addition, postpartum transmission of ts-1 is associated with an increased development of lymphoma in pups [2, 3].

Chakraborty et al. [4] have found that ts-1 integration leads to an overexpression of four genes associated with lymphoma in BALB/c mice infected by breast milk. This unique model has allowed us to demonstrate ts-1 retrovirus transmission via breast milk and study the molecular mechanism of the lymphoma development through natural

Table 1: Comparison between HIV and MoMuLV-ts-1 showing the similarities and differences between these two types of viruses.

Characteristics	HIV	ts-1
Retrovirus	+	+
Lentivirus	+	−*
Initial target for infection: CD4 cells	+	+
Depletion of T-lymphocytes	+	+
T-cell death via apoptosis	+	+
Inefficient transport of env precursor	+	+
Viral induced cytokine production	+	+
Polyclonal activation of B lymphocytes	+	+
Infection of astrocytes and microglia	+	+
Demyelination	+	+
Neuronal death	+	+
Wasting	+	+
Can cause lymphoma in some cases***	+	+
Horizontal transmission	+	−**
Mother-to-offspring transmission	+	+
Can cause disease	New born and adult	New born only

*MoMuLV-ts-1: mammalian gamma oncoretrovirus. **ts-1: No adult mouse can be infected; therefore, no sexual transmission occurs. ***: Both HIV and ts-1 may indirectly cause lymphoma.

transmission of a retrovirus to the offspring. Our hypothesis is that viral integration into the host genome alters host gene expression leading to an abnormal mRNA expression and abnormal protein production. These abnormal proteins alter the centrosome function during the cell cycle resulting in lymphomagenesis. This paper will explore two related areas of ts-1 research: ts-1 as a small animal model of perinatal retroviral transmission and ts-1 in lymphomagenesis.

2. Part I. MoMuLV-ts-1 as a Small Animal Model

2.1. The Similarities and Differences between HIV and MoMuLV-ts-1. HIV shares many characteristics with ts-1. These include CD4 cell targeting, secondary infections, neurodegenerative diseases, macrophage and CD4 cell infection, immunodeficiency, neurotropism, CD4 cell depletion, wasting, lymphomas, and perinatal transmission. However, the mechanism of entry of these two viruses are different. The Moloney murine leukemia virus (MoMuLV) ts-1 is a temperature sensitive mutant virus [5–7] first isolated by propagating MoMuLV in a thymus bone marrow cell line (TB) taken from CFW/D mice. This virus has a defect in the intracellular processing of the envelope precursor polyprotein (Pr80env) at the restrictive temperature [6–9]. Like HIV, MoMuLV ts-1 infects CD4 cells, with subsequent CD4 depletion and a resulting immunodeficiency (see Table 1) [10]. ts-1 is a murine gamma retrovirus that can induce T-cell lymphomas in susceptible strains of mice after a long incubation period [11].

As a simple retrovirus, ts-1 has only three genes (gag, env, and pol). Infection with ts-1 results in an AIDS-like syndrome in mice similar to HIV infection in humans [10, 12–14]. The most important characteristic of ts1 to our study is its effects on MTCT by breastfeeding in mice [2–4]. About 97% of uninfected neonatal mice that suckle from an infected mother develop clinically symptomatic ts-1 infection [3, 4]. Newborn BALB/c mice infected with ts1 virus suffer from neurodegenerative disease resulting in hind limb paralysis and immunologic disease characterized by severe thymic atrophy associated with immunodeficiency due to destruction of T-lymphocytes, and generalized body wasting [7, 15]. Infectivity of ts-1 is significantly related to its temperature sensitivity [7], and can replicate optimally at a permissive temperature of 34°C [16]. This may explain why the ts-1 virus can produce hind limb paralysis in newborn mice and not in the adult, because the body temperature of the newborn mice is lower (∼34°C) than that of adult (38.4°C) [17]. The uniqueness of the ts-1 among other murine retroviruses is that it can cause degenerative diseases in mice similar to HIV in humans, by affecting both the central nervous system (CNS) and the immune system. Infected T-lymphocytes have impaired function [14, 18].

The murine ts-1 model has been extensively used as a small-animal model for retrovirus-induced neurodegenerative disease [19]. Oxidative stress has been suggested as a major mechanism for ts1-induced neurodegeneration and T-cell loss in infected newborn mice [20]. The U3 region of ts-1 controls the pathogenicity and targets cell type [21].

Transfer of humoral immunity to ts-1 can be passed from mother to baby via breast milk and can provide protection from neurodegenerative and immunologic disease in neonatal mice [22]. Chakraborty et al. [23], have further developed this murine model to study the perinatal transmission of ts-1. Infected mothers can transmit the ts-1 virus vertically to offspring. They further demonstrated that mother-to-offspring transmission via breast milk can occur at nearly a 100% incidence [4], and can cause lymphoma when control pups suckle from ts-1 infected mothers. The pattern of proviral ts-1 integration sites observed in these lymphoma tissues correlates with the upregulation of mRNA expression of candidate genes that may contribute to lymphomagenesis [4].

2.2. Mother-to-Child Transmission of HIV by Breastfeeding. MTCT of HIV can occur *in utero, intrapartum,* or through breastfeeding. Current strategies aimed at decreasing MTCT of HIV have focused mainly on *in utero* and *intrapartum* transmission. Prophylaxis with highly active antiretroviral therapy (HAART) during pregnancy and through delivery has substantially reduced the rate of MTCT of HIV at birth [24]. Avoidance of breastfeeding by HIV-infected mothers is the norm in developed countries throughout the world [25]. However, for socioeconomic and cultural reasons, such avoidance is not an acceptable or viable alternative for many HIV-infected women in Africa and other resource poor areas worldwide [26]. In these areas, breastfeeding is the best and only source of infant nutrition, and safe alternatives do not exist because of inadequate supplies of formula and the lack of clean water. Breastfeeding, therefore, accounts for a substantial proportion of the overall MTCT

Table 2: Percentages of changes in spleen, lymph node, and thymus due to MTCT of ts-1 virus by breastfeeding in control and infected pups.

Group	Description	Sample size (n)	Splenomegaly	Lymph node hypertrophy	Thymic hypertrophy
1	Control pup nursed by infected surrogate mother	93	95.7%	79.1%	95.7%
2	Infected pup nursed by infected surrogate mother	94	92.6%	56.5%	92.6%
3	Infected pup nursed by control surrogate mother	108	46.3%	25.9%	46.3%
5	Infected pup nursed by biological mother	88	89.8%	61.4%	89.8%
4 & 6	Control—no infection	154	1.9%	0.0%	5.8%

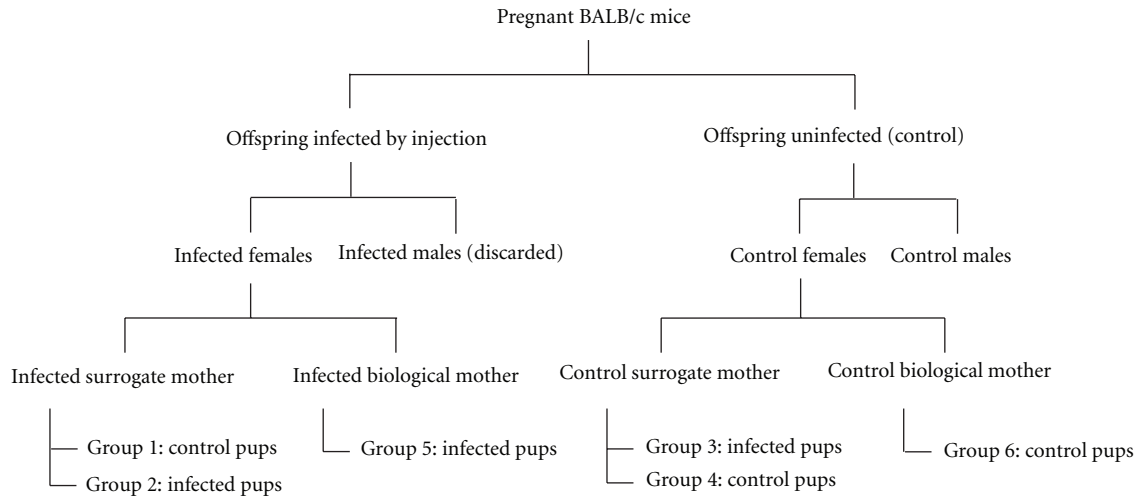


Figure 1: Experimental plan for ts-1 breast milk transmission studies.

rate in developing countries. Breastfeeding for 15 months accounts for 38% of the overall MTCT rate [27], and if continued for 24 months, it accounts for 44% [28]. Richardson et al. [29] reported that if an infant ingests one liter of breast milk then the probability of having the HIV-1 infection is similar to that of heterosexual transmission. Breast milk transmission depends on high maternal viral load and status of immunodeficiency with mastitis and duration of breastfeeding increasing the rate of HIV infection [29]. Breastfeeding by HIV positive women can reverse the benefits made in reducing perinatal HIV transmission through the use of HAART during pregnancy and/or labor. For example, in the Petra study of MTCT of HIV [30], the benefits of perinatal HAART prophylaxis were lost in the breastfeeding group. In order to prevent milk-borne HIV, HAART must be given throughout the breastfeeding period, which is rarely a feasible option in developing countries.

Studies in primates with HIV-2 [31], simian immunodeficiency virus (SIV) and SHIV (SIV expressing HIV envelope) [32, 33] have shown that primate lentiviruses can be transmitted orally. After milk is swallowed, gastric acid may inactivate some virus, but the buffering capacity of milk may allow the remaining virus to enter into the lumen of the gut, where there is extensive lymphoid tissue as a target for HIV. Mixed feeding (breastmilk and formula) carries a significantly increased risk of HIV transmission, because formula foods increase gut permeability [34]. These data suggest that the intestine could be a site of HIV entry in breast milk transmission, in addition to the oropharyngeal cavity. In areas without clean water, WHO recommends exclusively breastfeeding to avoid fatal diarrhea and the increased risk of HIV infection related to mixed feeding of formula and breast milk [35].

Several methods have been studied to decrease MTCT of HIV in areas where breastfeeding is a necessity. These include wet nursing by an HIV-negative woman, heat-treating the mother's milk with Holder pasteurization (62.5° for 30 min), leaving milk at room temperature for 30 minutes to allow milk lipase to inactivate HIV, adding microbicides to milk and standing for 5–10 minutes, or getting milk from a human milk bank [35, 36]. Due to high cost, obtrusiveness, inaccessibility, and/or inconvenience, none of these options are practical in developing countries where breastfeeding remains the only viable option for infant feeding and thus a major route of vertical HIV transmission. Therefore, a prophylaxis is urgently needed. However, in order to develop an effective prophylaxis, the exact mechanism of breast milk transmission of retrovirus must be clearly determined. In-depth study of the pathophysiology of retroviral transmission via breast milk using an animal model such as our ts-1 model will provide mechanistic insights that can translate into effective human therapies.

2.3. Breast Milk Transmission of MoMuLVts-1 in BALB/c Mice. In the study of viral transmission by breast milk, use of

Table 3: Correlation between mothers viral load and selected pup body and organ weights.

Group	Mothers				Pups				
	Treatment	Sample size (n)	Viral load	Body weight	Sample size (n)	Body weight	Spleen weight	Lymph node weight	Thymus weight
1	Infected surrogate mother/control pup	6	1.657E+05	19.58	32	24.14	0.74	0.31	0.31
2	Infected surrogate mother/infected pup	11	2.612E+05	21.24	57	23.44	0.78	0.34	0.19
3	Uninfected surrogate mother/infected pup	6	0.000E+00	25.24	38	28.40	0.43	0.08	0.08
4	Uninfected surrogate mother/infected pup	15	0.000E+00	25.99	80	28.31	0.11	0.00	0.04
5	Infected biological mother	17	2.332E+05	21.25	66	24.18	0.59	0.22	0.16
6	Uninfected biological mother	15	0.000E+00	27.00	81	28.44	0.10	0.00	0.05

Total number of mothers: 67; total number of offspring males: 158; total number of offspring females: 196; total number of animals 421.

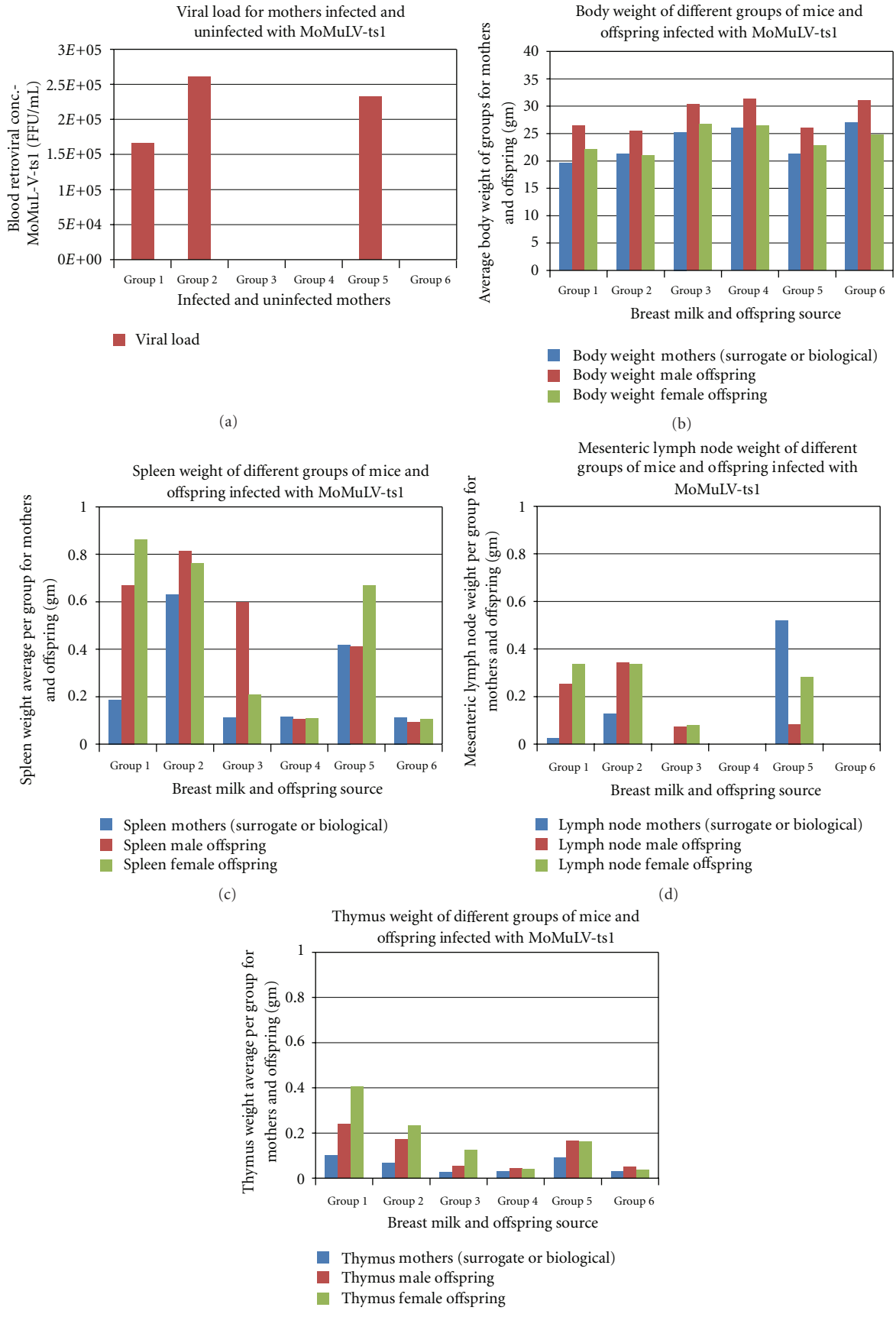


Figure 2: Viral load comparisons of infected and control mothers. Body, spleen, lymph node, and thymus weight comparisons for mothers and offspring of different groups.

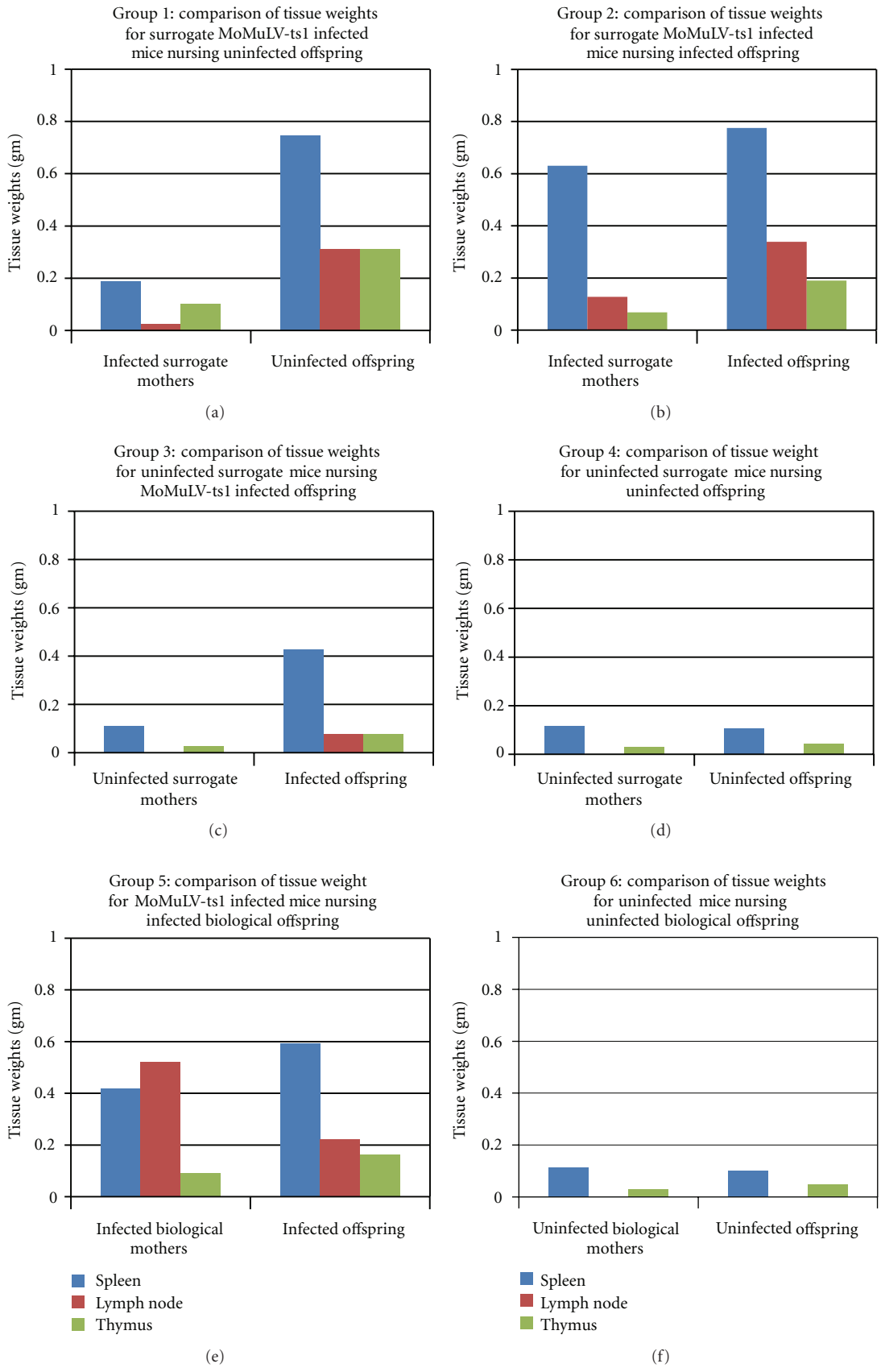


Figure 3: Tissue weight of mothers and offspring of each of 6 groups.

Table 4: Flow cytometric Analysis of CD4+, CD8+, and CD19 Cells.

		Flow Cytometric analysis		
		CD4 (T-cell)	CD8 (T-cell)	CD19 (B-cell)
Blood	Control	15.4 ± 10.6	4.4 ± 2.9	2.6 ± 1.2
	Infected (no lymphoma)	5.1 ± 4.2	1.0 ± 0.7	0.5 ± 0.6
	Infected (with lymphoma)	3.2 ± 2.4	1.5 ± 1.2	1.7 ± 1.3
Spleen	Control	13.1 ± 4.7	4.9 ± 2.0	22.6 ± 7.1
	Infected (no lymphoma)	12.5 ± 0.8	4.6 ± 0.3	10.1 ± 2.4
	Infected (with lymphoma)	2.8 ± 0.6	1.8 ± 1.2	2.2 ± 1.2
Thymus	Control	15.6 ± 5.6	16.6 ± 8.5	0.9 ± 0.2
	Infected (no lymphoma)	25.3 ± 33.3	1.2 ± 0.1	2.0 ± 1.4
	Infected (with lymphoma)	8.6 ± 9.2	8.9 ± 13.5	0.4 ± 0.5
Lymph Node	Control	(NA)	(NA)	(NA)
	Infected (no lymphoma)	(NA)	(NA)	(NA)
	Infected (with lymphoma)	11.3 ± 8.2	4.3 ± 3.6	8.6 ± 7.0

(NA): Not Available.

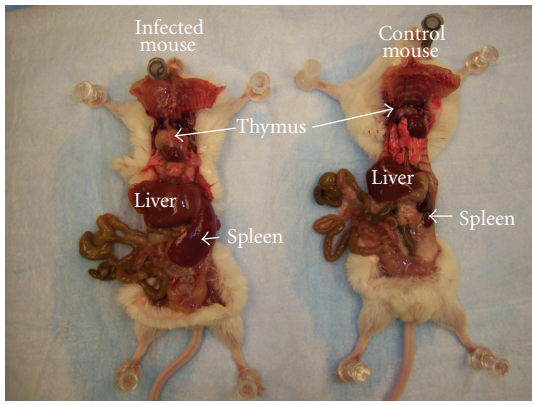


Figure 4: Organomegaly in infected versus control mice.

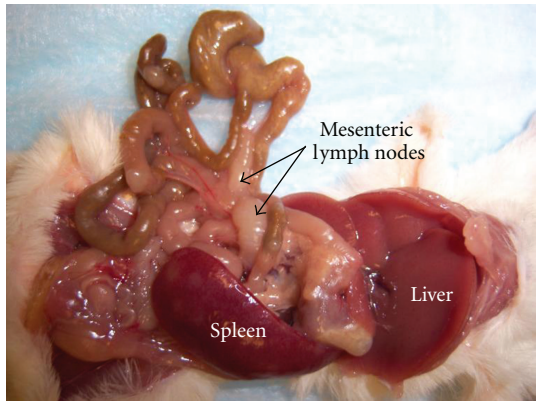


Figure 5: Manifestations of MoMuLV-ts-1 retroviral infection on different organ system.

a small animal model with a well-characterized immune system, such as the mouse, is ideal. As mentioned above, infection with ts-1 results in an AIDS-like syndrome. When neonatal BALB/c mice less than 10 days old are injected intraperitoneally with ts-1, clinically apparent infection

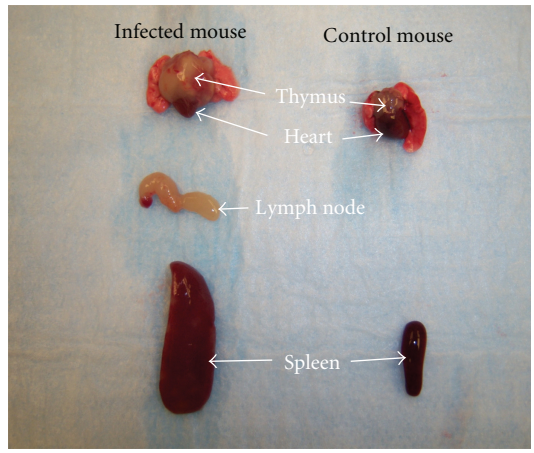
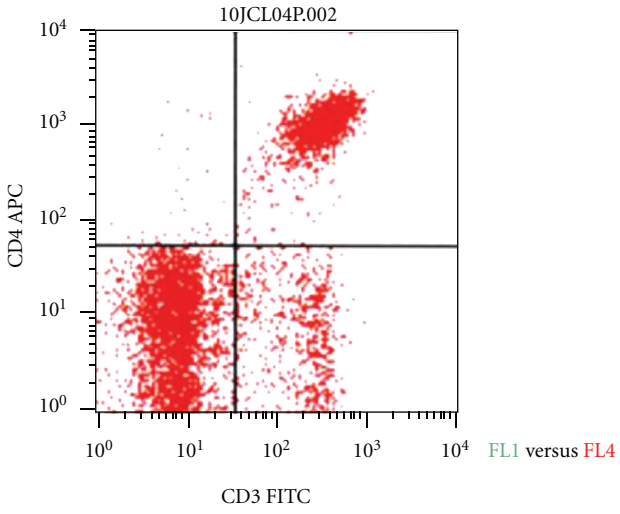


Figure 6: Excised organs from infected versus control mouse.

develops within 10 weeks. This clinical syndrome in mice is characterized by progressive bilateral hind limb paralysis, severe body wasting, and immunodeficiency [15]. Milder clinical illness can be induced by varying the dose of virus injected and/or the time of viral inoculation up to 10 days of age. CD4 cells are depleted by apoptosis, yielding AIDS-like clinical manifestations [12–14].

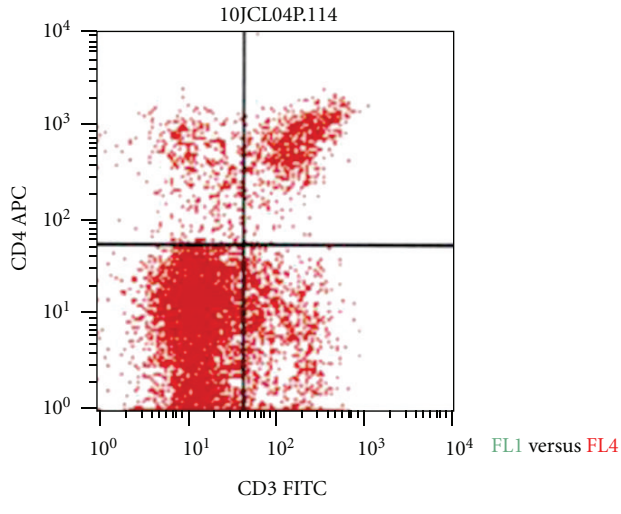
Several unique characteristics of this virus determine the experimental manner in which it is evaluated. The infectivity of this virus is strain specific and dependent upon the age of the mouse at the time of exposure, but the temperature sensitive nature of the virus does not affect its *in vivo* infectivity [15, 37]. BALB/c mice are a susceptible strain, with clinical diseases developing in 98% of mice by week 10 after intraperitoneal injection. Viral injection of mice after ten days of age does not result in clinically detectable disease but does result in antibody production [19, 38]. Perinatal transmission of ts-1 occurs *in utero, intrapartum*, and via breastfeeding [2, 3]. Breastfeeding is a highly effective route of MTCT in this model: 95% to 99% of uninfected neonatal



Quad	Events	Gated (%)	Total (%)	X mean	Y mean
UL	39	0.66	0.24	12.3	337.3
UR	1406	23.69	8.8	362.75	1156.95
LL	3669	61.83	22.97	8.93	10.49
LR	820	13.82	5.13	226.42	9.21

CD_4 cells from control offspring

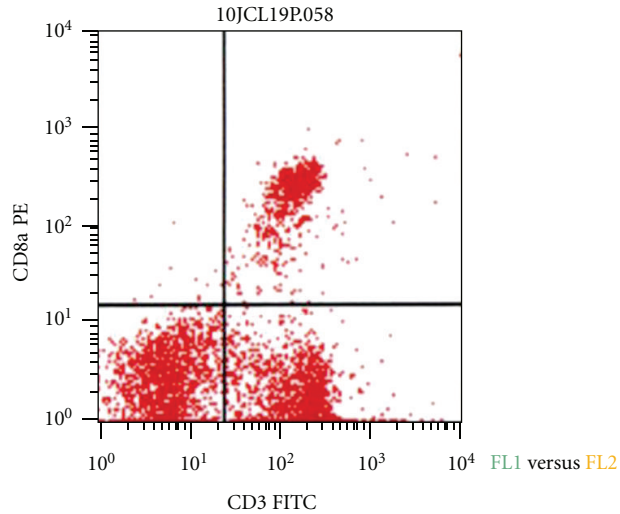
(a)



Quad	Events	Gated (%)	Total (%)	X mean	Y mean
UL	415	5.42	1.26	16.8	488.74
UR	697	9.11	2.11	206.42	740.79
LL	5473	71.54	16.55	14.73	10.44
LR	1065	13.92	3.22	144.47	9.11

CD_4 cells from infected offspring

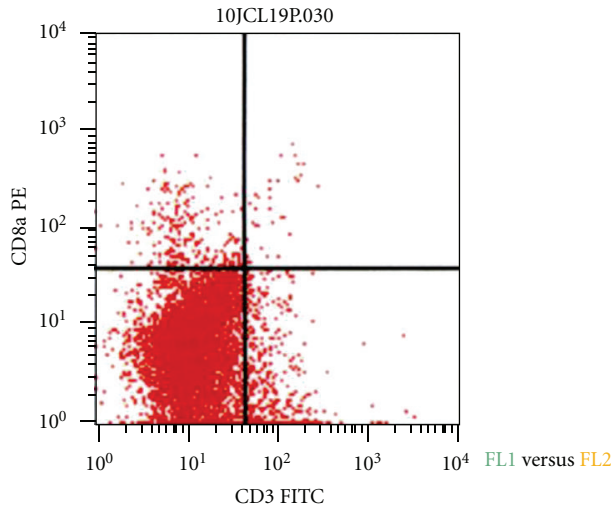
(b)



Quad	Events	Gated (%)	Total (%)	X mean	Y mean
UL	11	0.2	0.11	11.21	31.72
UR	781	14.39	7.66	187.62	232.14
LL	1615	29.75	15.83	7.02	3.27
LR	3022	55.66	29.63	220.23	1.67

CD_8 cells from control offspring

(c)



Quad	Events	Gated (%)	Total (%)	X mean	Y mean
UL	231	3.12	2.06	14.35	108.22
UR	42	0.57	0.37	94.03	201.36
LL	6511	88.06	58.03	13.73	8.04
LR	610	8.25	5.44	125.47	5.18

CD_8 cells from infected offspring

(d)

Figure 7: Examples of the flow cytometric analysis showing frequency of CD4+ and CD8+ cells from control and infected offspring. Samples were tagged with CD3 FITC, CD4 APC and CD3 FITC, CD8 PE. Upper left quadrant (UL) shows naïve lymphocytes. Upper right quadrant (UR) shows CD3+/CD4+ or CD3+/CD8+ frequency for cells positive for both CD3 and CD4 or CD3 and CD8 markers. Lower left quadrant (LL) shows CD3−/CD4− or CD3−/CD8− frequency for cells negative for both CD3/CD4 or CD3/CD8 markers. Lower right quadrant (LR) shows cells positive for CD3 markers only.

Table 5: Retrovirus genera and type of species affected.

Classification of retroviruses		
Genus	Species	Examples
Alpharetrovirus	Birds	Avian leukosis viruses (AVL), Rous sarcoma virus (RSV)
Betaretrovirus	Mice, primates, and sheep	Mouse mammary tumor virus (MMTV), Mason-Pfizer monkey virus (MPMV), and Jaagsiekte sheep retrovirus (JSRV)
Gammaretrovirus	Mice, cats, primates, and birds	Murine leukemia virus (MLV), feline leukemia viruses, gibbon ape leukemia virus, reticuloendotheliosis virus, and xenotropic murine retrovirus (xMRV)
Deltaretrovirus	Cattle and primates	Bovine leukemia virus, human T-lymphotropic virus
Epsilonretrovirus	Fish	Walleye dermal sarcoma virus
Lentivirus	Primates, sheep, cats, and horses	Human immunodeficiency virus (HIV), simian immunodeficiency virus (SIV), Maedi/visna virus, feline immunodeficiency virus (FIV), and equine infectious anemia virus
Spumavirus	Primates, cats, and cattle	Human foamy virus (HFV), simian foamy virus (SFV), feline foamy virus, and bovine foamy virus

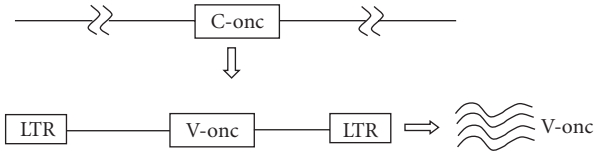


Figure 8: Acute transforming virus, capture of a c-onc and over-expression of v-onc by provirus.

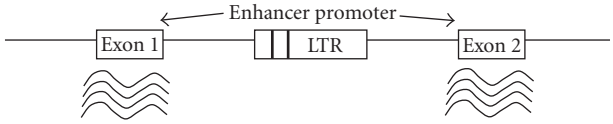


Figure 9: Enhancer of promoter insertion either upstream or downstream of growth controlling cellular genes.

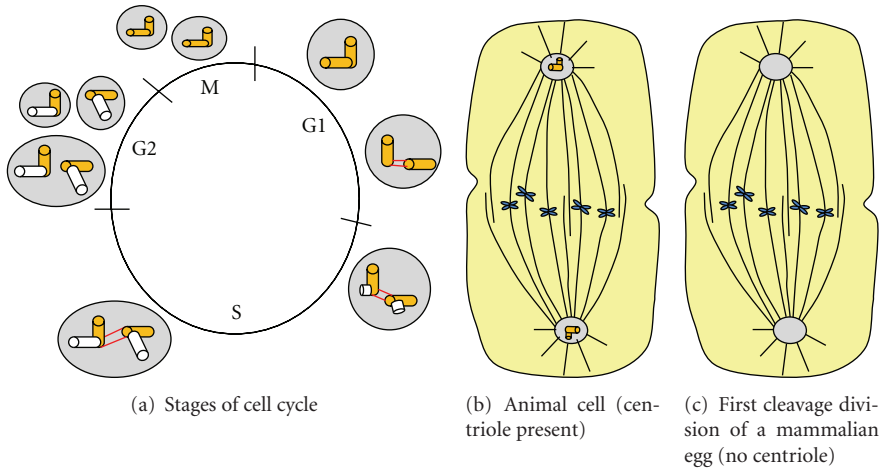
mice, if suckled from an infected mother, develop clinically symptomatic ts-1 infection [3, 4].

Our laboratory was the first to clearly demonstrate that breast milk transmission in offspring of ts-1 infected BALB/c mice causing AIDS like condition. We have established a mouse model for MTCT for MoMuLV ts-1 for in utero, intrapartum, and postpartum (breastfeeding) routes of transmission [2–4, 23]. The following information provide some murine model data for retroviral transmission via breastfeeding. Pregnant BALB/c female mice delivered pups which were divided into experimental and control groups. Seventy two hours after birth, the pups were injected intraperitoneally (ip) with 0.1 mL of 4.0×10^6 ffu/mL ts-1 virus and control pups were injected with 0.1 mL DMEM medium [2–4]. These mice were allowed to mate with uninfected males and produce offspring. Within 10–12 h after birth, offspring suckled either from control or ts1-infected surrogate or biological mothers (Figure 1). Tissue collection, histology, electron microscopy, flow cytometry, DNA extraction, PCR, and sequencing of viral DNA were performed. Four hundred twenty one mice were divided into 6 groups as described in our previous paper [3]. Pups from the ts-1 control mothers suckled from the infected surrogate mothers (Group 1), pups from the infected mothers suckled from the infected surrogate mothers (Group 2), or from infected biological mothers (Group 5). In Group 3, infected pups suckled from the control mothers. Groups 4 and 6 were controls. Group 4 control pups suckled from the control surrogate mothers, and Group 6, control pups suckled from their control biological mothers.

The rate of postpartum ts-1 transmission in this study was almost 100%. This rate is much higher than the MTCT of HIV in infants via breastfeeding (about 20%–30%), which is advantageous in an experimental animal model. Maternal viral load appear to correlate with development of lymphoma (Table 3; Figures 2 and 3), but further studies are needed to verify this. The figures and tables listed below show some of the gross morphology and flow cytometric data. Significant and consistent increases in weights and change in gross morphology of spleen, lymph node, and thymus (Tables 2 and 3; Figures 4, 5, and 6) are readily apparent. Our flow cytometric data shows decreases in CD4 and CD8 (Figure 7) cell population in the advanced stages of lymphoma development. In addition, decreasing numbers of CD8 and B-cells have been shown (Table 4) in the presence of clinically apparent immunodeficiency and wasting.

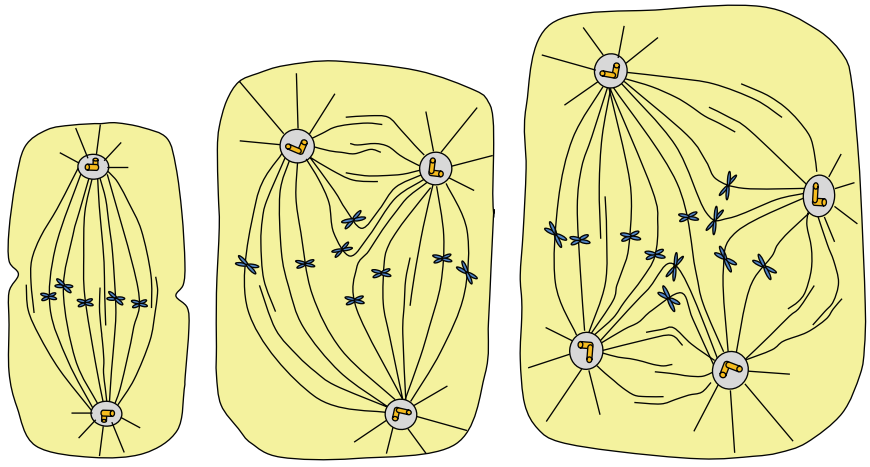
3. Part II. ts-1 and Lymphomagenesis

3.1. Retrovirus Induced Cancer. The first reports of retroviruses associated with cancer occurred in the early 20th century when an avian erythroblastosis virus (AEV) was isolated from spontaneous erythroleukemia in a chicken [39]. Shortly after that, Peyton Rous demonstrated that chicken sarcomas were infectious and could induce tumors when transmitted into healthy birds [40]. These novel observations were followed by multiple reports of retroviruses isolated from a broad range of mammals such as rodents, cats, sheep, and cows in association with both malignancies and immunodeficiencies. The first human retrovirus, human T-lymphotropic virus type 1 (HTLV-1), was isolated in 1980 [41] and has been shown to induce adult T-cell leukemia (ATL) [42].



(a) Stages of cell cycle (b) Animal cell (centriole present) (c) First cleavage division of a mammalian egg (no centriole)

Figure 10: This figure shows the centriole-centrosome division during cell cycle (a), presence of both centriole and centrosome in the animal cell (b), and absence of centriole in the first cleavage division of a mammalian egg (c).



Normal division (a) Tripolar (b) Tetrapolar (c)

Figure 11: Normal and abnormal cell division due to abnormal centriole-centrosome division. As a result, giant cells with multiple nuclei may be present in a malignant cell.

Retroviruses are classified into seven genera. Oncogenic retroviruses (retroviruses that induce tumorigenesis) belong to the following genera (Table 5) [43, 44].

Oncogenic retroviruses which induce tumors can be divided into two classes: acute and slow transforming viruses. Acute transforming retroviruses induce polyclonal tumors within 2 to 3 weeks after infection of the host. These retroviruses induce tumors through acquisition and overexpression of cellular proto-oncogenes that have captured virus (Figure 8) [45]. An example is v-Abl in the Abelson Murine Leukemia Virus [46].

In contrast, slow transforming retroviruses induce mono- or oligoclonal tumors with a longer latency of several months. These types of retroviruses do not carry viral oncogenes and can cause tumors by activating cellular proto-oncogenes close to the proviral DNA integration site on the host genome (Figure 9).

Elements in the proviral genome that regulate the viral transcript also act at common integration sites (*cis)* on cellular gene transcripts. Depending on whether the provirus integrates into the genes or in the vicinity of the genes, these elements can enhance or disrupt normal transcription, and thus induce oncogenic mutations. These classes of retroviruses have been found to induce tumors in many animals, including birds (ALV and REV), and mice (MLV); [45]. In addition to these two general transformation groups, a small number of retroviruses induce tumors by expression of their own oncogenic proteins. For example, human T-cell leukemia virus types 1 and 2 (HTLV-1 and HTLV-2, resp.) induce adult T-cell immortalization and leukemia in human by expression of viral Tax protein. Tax has no cellular homologue, and it works in trans to disrupt cellular checkpoints and destabilize genome integrity [47] leading to transformations that directly cause human cancer [48].

Table 6: Gene expression analysis of the spleen tissues were used to investigate the centriole-centrosome pathway for infected versus control mice. *tacc3* showed the highest upregulation with 30.13-fold increase, much more than our previous report [4]. *tpx2* and *p53* showed significant downregulation. During this investigation, *aurka* did not show significant upregulation. This is due to the variation in this group of six mice used in this study. However, a trend of upregulation of *aurka* is evident in 3 mice in column 2.

	Gene expression					
Mouse ID	*tacc3*	*aurka*	*ndel1*	*tpx2*	*p53*	*rhamm*
Control 1	(NA)	(NA)	1.18	(NA)	(NA)	(NA)
Control 2	(NA)	(NA)	0.17	(NA)	0.45	0.56
Control 3	2.32	0.8	0.42	0.91	0.66	0.75
Control 4	0.37	0.96	2.78	1.85	1.95	1.53
Control 5	0.85	0.96	0.57	1.19	0.93	1.17
Control 6	0.64	1.52	0.09	0.17	(NA)	(NA)
Control 7	0.82	0.76	1.81	0.88	(NA)	(NA)
SEM	**0.34**	**0.14**	**0.37**	**0.27**	**0.33**	**0.22**
Normalized avg. fold Exp.	**1.00**	**1.00**	**1.00**	**1.00**	**1.00**	**1.00**
Lymphoma 1	11.25	1.25	0.14	0.15	0.26	1.63
Lymphoma 2	(NA)	0.44	0.12	0.2	0.13	0.67
Lymphoma 3	(NA)	0.4	0.25	0.11	0.15	1.63
Lymphoma 4	53.53	2.41	0.2	0.15	0.71	0.88
Lymphoma 5	53.16	1.87	0.24	0.15	0.6	0.9
Lymphoma 6	2.57	(NA)	0.09	0.15	0.07	0.85
Avg Fold Exp.	**30.13**	**1.27**	**0.17**	**0.15**	**0.32**	**1.09**
SEM	**13.52**	**0.39**	**0.03**	**0.01**	**0.11**	**0.17**
$P \leq 0.05$	**0.04**	**0.53**	**0.07**	**0.01**	**0.05**	**0.75**

(NA): data not available.

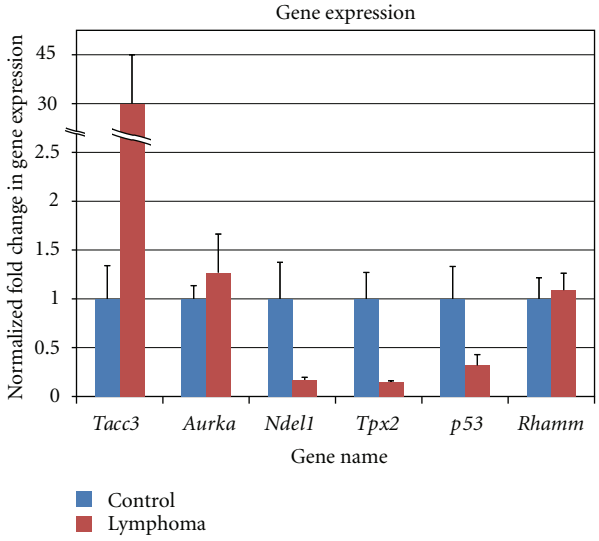


Figure 12: Graph of fold change in gene expression versus normalized control.

In AIDS-related lymphoma, patient studies indicate that oncogene activation by insertional mutagenesis of the HIV might be another mechanism by which HIV-1 can induce cancer [49, 50].

The release of the complete mouse genome sequence and the availability of reliable methods for isolation of proviral flanks have introduced the retroviral insertion mutagenesis screen in mice as a powerful procedure to identify genes contributing to tumorigenesis. Many oncogenes identified in these screens have given a valuable basis for better understanding the development of human cancer [45]. The MLV utilizes a slow transformation mechanism to induce leukemia or lymphoma in mice and is one of the retroviruses that provides an excellent model to identify and study the oncogenes involved in retrovirus-induced tumorigenesis.

3.2. Centrosomal Involvement in Cell Cycle and Cancer. Centriole-centrosome is the microtubular organization center (MTOC) and plays a major role during mitosis for chromosomal arrangement at the equatorial plane during metaphase and pulling the chromatids in two opposite spindle poles during anaphase. Spindles start to organize as soon as centrioles divide and two centrosomes are formed. The spindle microtubules act like railroad tracks on which the chromatids travel towards the poles with the help of several motor proteins including dynein and kinesin. However, the centriole is not an essential structure for many cells including first cleavage division of mammalian eggs [51]. Figure 10 describes the centrosome-centriole cycle in a typical animal cell at mitosis and the first cleavage division of a mammalian egg without the centriole. The centrosome—which is an accumulation of amorphous materials around the centriole—is present in most mammalian cells. In these

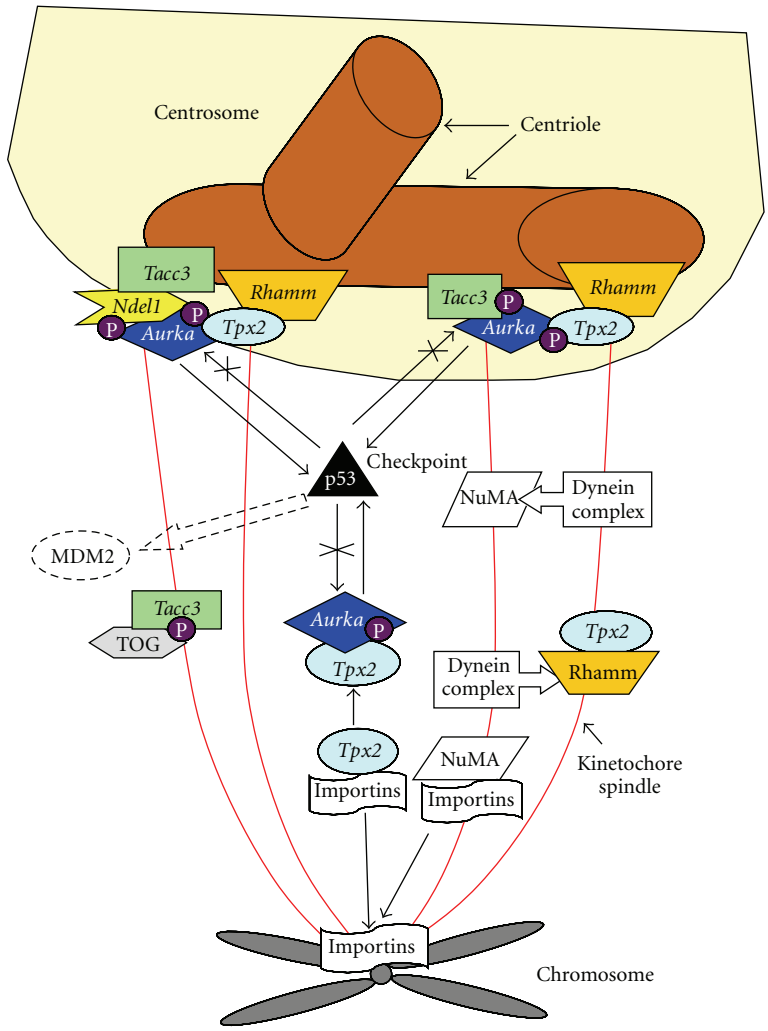


Figure 13: Pathway of Genes associated with spindle formation.

cells, the centrosome is an essential component in the development of spindle poles and progression to mitosis and cytokinesis. There are three types of spindles: kinetochore, interpolar, and astral. The kinetochore spindle extends from poles to chromosomes. The interpolar spindles run from pole to pole with no connection to the chromosomes, while the astral spindles run between centrosomes to the cell cortex. Thus, three types of spindles play different roles.

There are numerous centrosomal proteins and new proteins are being discovered every year. Although the centriole-centrosome system is essential for cell cycle and normal chromosomal segregation, little is known about the intricate details of this structure, which is extremely important in the understanding of cancer development and progression. For example, many cancers show abnormal mitosis leading to tetraploidy and aneuploidy. According to Wang et al. [52], many carcinomas may have either or both structural and numerical abnormalities; this occurs during early stage of tumorigenesis. This is also correlated with chromosomal instability and tumor progression. Numerical chromosomal abnormalities can also occur due to bipolar and tripolar or tetrapolar spindle formation (Figure 11).

Unequal segregation of chromosomes leads to multiploidy and/or aneuploidy. Viral gene insertion into the normal genome close to the centrosomal genes may lead to abnormal mRNA expression resulting in abnormal protein production and activity. For example, increases in *Tacc3* and *Aurora A kinase* (Aurka) can produce tumor like cells [53, 54].

3.3. Insertion of Viral Genome into the Mouse Genome Causing Abnormal Centrosomal Protein Production Leading to Lymphomagenesis. A large number of investigations have been carried out to study the development of lymphoma by MoMuLV, but so far, little data are available on the natural transmission of this retrovirus by breast milk causing lymphoma. Extensive studies are needed on breast milk transmitted retroviruses, since evidence exists that viruses such as HTLV-1 or Epstein-Barr virus (EBV) may be oncogenic and also have epidemiologic patterns indicating perinatal or early childhood transmission. The best studied of these viruses is EBV. Studies have shown that EBV and MLV gene insertion into the host genome alters gene

expression leading to carcinogenesis [55–59]. We have used inverse PCR (I-PCR), DNA cloning, sequencing, and quantitative reverse transcriptase-PCR (qRT-PCR) to study the viral integration into mouse genome. Tissue samples from spleen and lymph nodes were used for the genomic studies [4]. Using the primary PCR product as template, secondary PCR was performed. After the PCR bands were obtained, cloning was done and colonies with viral inserts were located and analyzed. The differential expression of candidate genes was obtained by using quantitative real-time PCR. Gene specific primers were designed using Primer Express software. GAPDH was used as "housekeeping" gene to confirm amplification factor for each PCR product melt-curve analysis process. RNA expression levels were calculated by using ddCt method. Gene expression were recorded by mRNA expression and reported as "fold change". Statistical analyses were performed by using statistical package of the social sciences (SPSS). 35 genes were selected for mRNA expression based on preselected criteria. Table 6 and Figure 12 show the change in mRNA expression of six genes *Tacc3* shows the highest upregulation with 30.13-fold increase, much more than our previous report [4].

How are changes in gene expression associated with lymphoma in this murine model? In our mouse model, about 50% of pups develop lymphoma. This high incidence of lymphoma may be due to the genomic integration of viral DNA into the mouse genome causing changes in nearby genes. While viral gene insertion appears to be random, in pups that develop lymphoma, there is a predilection for viral insertion near genes involved in spindle formation. One of the mechanisms of lymphoma development, therefore, may be the alteration in spindle assembly-disassembly pathway. Up or down regulation of these genes may occur, breaking the balance of protein production, and causing abnormality in spindle formation, chromosomal segregation, and subsequent lymphoma production. Although there are many genes involved in the spindle assembly pathway, we have studied 6 genes predominately associated with viral genomic integration in mice with lymphoma including *tacc3, aurka, tpx2, rhamm, ndel1* and *p53*. Of these 6 genes, we have observed the highest fold increase of *tacc3* followed by *aurka*. Figure 13 shows the proteins (gene product) associated with spindle formation.

All tumor samples showed increases in mRNA expression of *tacc3* gene compared to the control group. Out of the 6 genes reported in this study, two were upregulated, including *tacc3* and *aurka.* As previously reported, *tacc3* showed the highest mRNA expression levels with an average of 9.16-fold increase [4]. *tacc3* is associated with centrosome and microtubule-associated proteins that are essential for mitotic spindle formation [60] and have been associated with dysfunction in a variety of tumors. *Tacc3* was also identified as a novel prognostic marker in nonsmall cell lung cancer [61]. Dysregulation of T*acc3* proteins have also been related to ovarian cancer [62]. Schnieder et al. [63] demonstrated the important role of T*acc3* in spindle assembly and cellular survival, thus introducing it as a potential therapeutic target in cancer cells. Deficiency of *Tacc3* leads to *p53*-mediated apoptosis [64]. Therefore, overexpression of *tacc3*, as observed in our mouse model may cause the downregulation of *p53*, causing inhibition of apoptosis, thus leading to lymphoma.

Aurka connects microtubules to kinetochore and phosphorylates T*acc3,* leading to its localization to spindle microtubules during metaphase and promoting its growth from centrosomes. *Aurka* is essential for accurate chromosome segregation. Overexpression of *Aurka* leads to spindle defects, aneuploidy, and tumor formation. *Tpx2* and *Rhamm* helps with stable bipolar spindle formation through its association with *Aurka* [65–67]. Decrease in expression of these proteins may cause spindle fragmentation, while increased expression may cause disorganized, multipolar spindles coupled with inability to appropriately align and segregate chromatids. *Ndel1* has a high affinity for T*acc3,* and its disruption decreases centrosome targeting of T*acc3* [68]. *p53* is a cell-cycle checkpoint protein. DNA damage generally results in phosphorylation of *p53* leading to its dissociation from *MDM2* and acts as a transcription factor, arresting the cells in the G1 phase of the cell cycle. Mutated or downregulated *p53* results in continued proliferation in damaged cells and subsequent development of cancer. Upregulation of *aurka* and *tacc3* and downregulation of *p53* are consistent with abnormal cell division, aneuploidy, and tumorigenesis. Downregulation of *tpx2* is associated with abnormal spindle formation with the presence of giant cells and the absence of mitotic figures in histological specimens of lymphoma tissue. However, spindle pathway disruption is not the only mechanism by which malignancies develop or progress, but it may be an important factor for breast milk-transmitted retrovirus-induced cancer in the ts-1 mouse model.

4. Conclusion

A well-developed animal model can be extremely important in the study of viral-induced cancer and immunodeficiency. Although there are obvious differences between murine and human cells, an appropriate mouse model can provide important clues to the possible pathways of molecular mechanisms of retroviral transmission and cancer development and progression. While this paper reviews ts-1 as a small animal model of perinatal HIV transmission and ts-1 in lymphomagenesis, it also raises a number of questions that need further study. We believe that this is a unique murine model for the study of retrovirus-induced lymphomagenesis for multiple reasons. Mice have been used extensively for genetic, physiological, biochemical, immunological, endocrinological, and reproductive research and an enormous body of background data—including the entire murine genome—is already available. In our unique murine model, breast milk transmission is near 100%, but lymphoma development occurs in only 50% of infected pups across all litters; therefore, an ideal internal control exists in which to study causative genetic factors. Study of this model may contribute to the understanding of molecular mechanisms of spindle formation, chromosomal segregation, and interactions between many centrosomal proteins and may help clarify some of the cellular abnormalities leading to

cancer. Some of these proteins and their inhibitors can be used for diagnostic and/or therapeutic development.

Acknowledgment

This work was supported by F. M. Douglas Foundation of St. Vincent Medical Center.

References

[1] UNAIDS and WHO, 2008, AIDS epidemic update, Joint United Nations Program on HIV/AIDS.

[2] J. Duggan, H. Okonta, and J. Chakraborty, "Vertical transmission of a murine retrovirus, ts1," *Viral Immunology*, vol. 17, no. 2, pp. 266–278, 2004.

[3] J. Duggan, H. Okonta, and J. Chakraborty, "Transmission of Moloney murine leukemia virus (ts-1) by breast milk," *Journal of General Virology*, vol. 87, no. 9, pp. 2679–2684, 2006.

[4] J. Chakraborty, B. Hussein, H. Bagalb et al., "Retroviral gene insertion in breast milk mediated lymphomagenesis," *Virology*, vol. 377, no. 1, pp. 100–109, 2008.

[5] P. K. Y. Wong, L. J. Russ, and J. A. McCarter, "Rapid, selective procedure for isolation of spontaneous temperature-sensitive mutants of Moloney leukemia virus," *Virology*, vol. 51, no. 2, pp. 424–431, 1973.

[6] P. K. Y. Wong and J. A. McCarter, "Studies of two temperature sensitive mutants of Moloney murine leukemia virus," *Virology*, vol. 58, no. 2, pp. 396–408, 1974.

[7] P. K. Y. Wong, M. M. Soong, R. MacLeod, G. E. Gallick, and P. H. Yuen, "A group of temperature-sensitive mutants of Moloney leukemia virus which is defective in cleavage of env precursor polypeptide in infected cells also induces hind-limb paralysis in newborn CFW/D mice," *Virology*, vol. 125, no. 2, pp. 513–518, 1983.

[8] P. K. Y. Wong and J. A. McCarter, "Genetic studies of temperature sensitive mutants of Moloney murine leukemia virus," *Virology*, vol. 53, no. 2, pp. 319–326, 1973.

[9] P. K. Y. Wong, C. Knupp, P. H. Yuen, M. M. Soong, J. F. Zachary, and W. A. F. Tompkins, "ts-1, a paralytogenic mutant of Moloney murine leukemia virus TB, has an enhanced ability to replicate in the central nervous system and primary nerve cell culture," *Journal of Virology*, vol. 55, no. 3, pp. 760–767, 1985.

[10] S. Clark, J. Duggan, and J. Chakraborty, "ts1 and LP-BM5: a comparison of two murine retrovirus models for HIV," *Viral Immunology*, vol. 14, no. 2, pp. 95–109, 2001.

[11] P. H. Yuen and P. F. Szurek, "The reduced virulence of the thymotropic Moloney murine leukemia virus derivative MoMuLV-TB is mapped to 11 mutations within the U3 region of the long terminal repeat," *Journal of Virology*, vol. 63, no. 2, pp. 471–480, 1989.

[12] K. Saha and P. K. Y. Wong, "ts1, a temperature-sensitive mutant of Moloney murine leukemia virus TB, can infect both $CD4^+$ and $CD8^+$ T cells but requires $CD4^+$ T cells in order to cause paralysis and immunodeficiency," *Journal of Virology*, vol. 66, pp. 2639–2646, 1992.

[13] K. Saha, P. H. Yuen, and P. K. Y. Wong, "Murine retrovirus-induced depletion of T cells is mediated through activation-induced death by apoptosis," *Journal of Virology*, vol. 68, no. 4, pp. 2735–2740, 1994.

[14] K. Saha and P. K. Y. Wong, "T, not B, lymphocytes are required for immunodeficiency and paralysis induced by ts1, a mutant of Moloney murine leukemia virus-TB," *Virology*, vol. 183, no. 2, pp. 815–820, 1991.

[15] P. K. Y. Wong, G. Prasad, J. Hansen, and P. H. Yuen, "ts1, a mutant of Moloney murine leukemia virus-TB, causes both immunodeficiency and neurologic disorders in BALB/c mice," *Virology*, vol. 170, no. 2, pp. 450–459, 1989.

[16] P. H. Yuen, D. Malehorn, C. Knupp, and P. K. Y. Wong, "A 1.6-kilobase-pair fragment in the genome of the ts1 mutant of Moloney murine leukemia virus TB that is associated with temperature sensitivity, nonprocessing of Pr80env, and paralytogenesis," *Journal of Virology*, vol. 54, pp. 364–373, 1985.

[17] C. G. Crispen Jr., *Handbook of the Laboratory Mouse*, Charles C Thomas, Springfield, Ill, USA, 1978.

[18] P. K. Y. Wong, E. Shikova, Y. C. Lin et al., "Murine leukemia virus induced central nervous system diseases," *Leukemia*, vol. 6, supplement 3, pp. 161S–165S, 1992.

[19] P. K. Y. Wong, "Moloney murine leukemia virus temperature-sensitive mutants: a model for retrovirus-induced neurologic disorders," *Current Topics in Microbiology and Immunology*, vol. 160, pp. 29–60, 1990.

[20] Y. Jiang, V. L. Scofield, M. Yan et al., "Retrovirus-induced oxidative stress with neuroimmunodegeneration is suppressed by antioxidant treatment with a refined monosodium alpha-luminol (Galavit)," *Journal of Virology*, vol. 80, no. 9, pp. 4557–4569, 2006.

[21] P. K. Y. Wong, P. F. Szurek, E. Floyd, K. Saha, and B. R. Brooks, "Alteration from T- to B-cell tropism reduces thymic atrophy and cytocidal effects in thymocytes but not neurovirulence induced by ts1, a mutant of Moloney murine leukemia virus TB," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 88, no. 20, pp. 8991–8995, 1991.

[22] K. Saha, D. Hollowell, and P. K. Y. Wong, "Mother-to-baby transfer of humoral immunity against retrovirus-induced neurologic disorders and immunodeficiency," *Virology*, vol. 198, no. 2, pp. 129–137, 1994.

[23] J. Chakraborty, S. Clark, H. Okonta, and J. Duggan, "A small animal model for mother-to-fetus transmission of ts1, a murine retrovirus," *Viral Immunology*, vol. 16, no. 2, pp. 191–201, 2003.

[24] S. Fiore and M. L. Newell, "Preventing perinatal transmission of HIV-1 infection," *Hospital Medicine*, vol. 61, no. 5, pp. 315–318, 2000.

[25] D. T. Dunn, M. L. Newell, A. E. Ades, and C. S. Peckham, "Risk of human immunodeficiency virus type 1 transmission through breastfeeding," *The Lancet*, vol. 340, no. 8819, pp. 585–588, 1992.

[26] C. Wendo, "Most Ugandan HIV-positive mothers insist on breastfeeding," *The Lancet*, vol. 358, no. 9282, p. 649, 2001.

[27] A. Coutsoudis, K. Pillay, L. Kuhn, E. Spooner, W.-Y. Tsai, and H. M. Coovadia, "Method of feeding and transmission of HIV-1 from mothers to children by 15 months of age: prospective cohort study from Durban, South Africa," *AIDS*, vol. 15, no. 3, pp. 379–387, 2001.

[28] H. M. Coovadia, "Prevention and treatment of perinatal HIV-1 infection in the developing world," *Current Opinion in Infectious Diseases*, vol. 13, no. 3, pp. 247–251, 2000.

[29] B. A. Richardson, G. C. John-Stewart, J. P. Hughes et al., "Breast-milk infectivity in human immunodeficiency virus

type 1-infected mothers," *Journal of Infectious Diseases*, vol. 187, no. 5, pp. 736–740, 2003.

[30] Petra Study Team, "The Petra trial: the efficacy of three short-course regimens of zidovudine and lamivudine (3TC) in preventing early and late transmission of HIV-1 from mother to child in an African setting: a randomized, double-blind placebocontrolled trial, conducted in South Africa, Tanzania and Uganda," *The Lancet*, vol. 359, pp. 1178–1186, 2002.

[31] A. M. Herz, M. N. Robertson, J. B. Lynch et al., "Viral dynamics of early HIV infection in neonatal macaques after oral exposure to HIV-2_{287} : an animal model with implications for maternal-neonatal HIV transmission," *Journal of Medical Primatology*, vol. 31, no. 1, pp. 29–39, 2002.

[32] R. M. Ruprecht, T. W. Baba, V. Liska et al., "Oral SIV, SHIV, and HIV type 1 infection," *AIDS Research and Human Retroviruses*, vol. 14, supplement 1, pp. S97–S103, 1998.

[33] R. M. Ruprecht, T. W. Baba, V. Liska et al., "Oral transmission of primate lentiviruses," *Journal of Infectious Diseases*, vol. 179, supplement 3, pp. S408–S412, 1999.

[34] N. C. Rollins, S. M. Filteau, A. Coutsoudis, and A. M. Tomkins, "Feeding mode, intestinal permeability, and neopterin excretion: a longitudinal study in infants of HIV-infected South African women," *Journal of Acquired Immune Deficiency Syndromes*, vol. 28, no. 2, pp. 132–139, 2001.

[35] R. Denison, "HIV and breastfeeding: what's a mother to do?" *World*, no. 126, pp. 5–7, 2001.

[36] WHO, *HIV Transmission Through Breast-Feeding: A Review of Available Evidence*, WHO, Geneve, Switzerland, 2004.

[37] P. K. Y. Wong, E. Floyd, and P. F. Szurek, "High susceptibility of FVB/N mice to the paralytic disease induced by ts1, a mutant of Moloney murine leukemia virus TB," *Virology*, vol. 180, no. 1, pp. 365–371, 1991.

[38] K. Saha and P. K. Y. Wong, "Protective role of cytotoxic lymphocytes against murine leukemia virus-induced neurologic disease and immunodeficiency is enhanced by the presence of helper T cells," *Virology*, vol. 188, no. 2, pp. 921–925, 1992.

[39] V. Ellermann and O. Bang, "Experimentelle Leukämie bei Hühnern. Zentralbl. Bakteriol. Parasite," *Infekt*, vol. 46, pp. 595–609, 1908.

[40] P. Rous, "A sarcoma of the fowl transmissible by an agent separable from the tumor cells," *Journal of Experimental Medicine*, vol. 13, pp. 397–411, 1911.

[41] B. J. Poiesz, F. W. Ruscetti, A. F. Gazdar, P. A. Bunn, J. D. Minna, and R. C. Gallo, "Detection and isolation of type C retrovirus particles from fresh and cultured lymphocytes of a patient with cutaneous T-cell lymphoma," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 77, no. 12, pp. 7415–7419, 1980.

[42] K. Barmak, E. Harhaj, C. Grant, T. Alefantis, and B. Wigdahl, "Human T cell leukemia virus type I-induced disease: pathways to cancer and neurodegeneration," *Virology*, vol. 308, no. 1, pp. 1–12, 2003.

[43] S. P. Goff, "Retroviridae: the retroviruses and their replication," in *Field Virology*, D. M. Knipe and P. M. Howley, Eds., vol. 2, Lippincott Williams & Wilkins, Philadelphia, Pa, USA, 4th edition, 2000.

[44] C. Voisset, R. A. Weiss, and D. J. Griffiths, "Human RNA "rumor" viruses: the search for novel human retroviruses in chronic disease," *Microbiology and Molecular Biology Reviews*, vol. 72, no. 1, pp. 157–196, 2008.

[45] A. G. Uren, J. Kool, A. Berns, and M. van Lohuizen, "Retroviral insertional mutagenesis: past, present and future," *Oncogene*, vol. 24, no. 52, pp. 7656–7672, 2005.

[46] S. K. Shore, R. V. Tantravahi, and E. P. Reddy, "Transforming pathways activated by the v-Abl tyrosine kinase," *Oncogene*, vol. 21, no. 56, pp. 8568–8576, 2002.

[47] K. T. Jeang and A. Gatignol, "Comparison of regulatory features among primate lentiviruses," *Current Topics in Microbiology and Immunology*, vol. 188, pp. 123–144, 1994.

[48] K. T. Jeang, "Retrovirology highlights a quarter century of HTLV-I research," *Retrovirology*, vol. 2, article 15, 2005.

[49] B. G. Herndier, B. T. Shiramizu, and M. S. McGrath, "AIDS associated non-Hodgkin's lymphomas represent a broad spectrum of monoclonal and polyclonal lymphoproliferative processes," *Current Topics in Microbiology and Immunology*, vol. 182, pp. 385–394, 1992.

[50] B. Shiramizu, B. G. Herndier, and M. S. McGrath, "Identification of a common clonal human immunodeficiency virus integration site in human immunodeficiency virus-associated lymphomas," *Cancer Research*, vol. 54, no. 8, pp. 2069–2072, 1994.

[51] L. Zamboni, J. Chakraborty, and D. M. Smith, "First cleavage division of the mouse zygot. An ultrastructural study," *Biology of Reproduction*, vol. 7, no. 2, pp. 170–193, 1972.

[52] Q. Wang, Y. Hirohashi, K. Furuuchi et al., "The centrosome in normal and transformed cells," *DNA and Cell Biology*, vol. 23, no. 8, pp. 475–489, 2004.

[53] E. A. Nigg, "Centrosome aberrations: cause or consequence of cancer progression?" *Nature Reviews Cancer*, vol. 2, no. 11, pp. 815–825, 2002.

[54] H. Zhou, J. Kuang, L. Zhong et al., "Tumour amplified kinase STK15/BTAK induces centrosome amplification, aneuploidy and transformation," *Nature Genetics*, vol. 20, no. 2, pp. 189–193, 1998.

[55] H. Fan, S. C. Kim, C. O. Chima et al., "Epstein-Barr viral load as a marker of lymphoma in AIDS patients," *Journal of Medical Virology*, vol. 75, no. 1, pp. 59–69, 2005.

[56] J. Li, H. Shen, K. L. Himmel et al., "Leukaemia disease genes: large-scale cloning and pathway predictions," *Nature Genetics*, vol. 23, no. 3, pp. 348–353, 1999.

[57] X. Liao, A. M. Buchberg, N. A. Jenkins, and N. G. Copeland, "Evi-5, a common site of retroviral integration in AKXD T-cell lymphomas, maps near Gfi-1 on mouse chromosome 5," *Journal of Virology*, vol. 69, no. 11, pp. 7132–7137, 1995.

[58] B. Scheijen, J. Jonkers, D. Acton, and A. Berns, "Characterization pal-1, a common proviral insertion site in murine leukemia virus-induced lymphomas of c-myc and Pim-1 transgenic mice," *Journal of Virology*, vol. 71, no. 1, pp. 9–16, 1997.

[59] M. S. Shin, T. N. Fredrickson, J. W. Hartley, T. Suzuki, K. Akagi, and H. C. Morse III, "High-throughput retroviral tagging for identification of genes involved in initiation and progression of mouse splenic marginal zone lymphomas," *Cancer Research*, vol. 66, no. 6, pp. 4419–4427, 2006.

[60] F. Gergely, C. Karlsson, I. Still, J. Cowell, J. Kilmartin, and J. W. Raff, "The TACC domain identifies a family of centrosomal proteins that can interact with microtubules," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 26, pp. 14352–14357, 2000.

[61] C. K. Jung, J. H. Jung, G. S. Park, A. Lee, C. S. Kang, and K. Y. Lee, "Expression of transforming acidic coiled-coil containing protein 3 is a novel independent prognostic marker in non-small cell lung cancer," *Pathology International*, vol. 56, no. 9, pp. 503–509, 2006.

[62] B. Lauffart, M. M. Vaughan, R. Eddy et al., "Aberrations of TACC1 and TACC3 are associated with ovarian cancer," *BMC Women's Health*, vol. 5, article 8, 2005.

[63] L. Schneider, F. Essmann, A. Kletke et al., "The transforming acidic coiled coil 3 protein is essential for spindle-dependent chromosome alignment and mitotic survival," *Journal of Biological Chemistry*, vol. 282, no. 40, pp. 29273–29283, 2007.

[64] R. P. Piekorz, A. Hoffmeyer, C. D. Duntsch et al., "The centrosomal protein TACC3 is essential for hematopoietic stem cell function and genetically interfaces with *p53*-regulated apoptosis," *EMBO Journal*, vol. 21, no. 4, pp. 653–664, 2002.

[65] P. J. LeRoy, J. J. Hunter, K. M. Hoar et al., "Localization of human TACC3 to mitotic spindles is mediated by phosphorylation on Ser558 by aurora A: a novel pharmacodynamic method for measuring aurora A activity," *Cancer Research*, vol. 67, no. 5362, pp. 5362–5370, 2007.

[66] R. Crane, B. Gadea, L. Littlepage, H. Wu, and J. V. Ruderman, "Aurora A, meiosis and mitosis," *Biology of the Cell*, vol. 96, no. 3, pp. 215–229, 2004.

[67] C. A. Maxwell, J. J. Keats, A. R. Belch, L. M. Pilarski, and T. Reiman, "Receptor for hyaluronan-mediated motility correlates with centrosome abnormalities in multiple myeloma and maintains mitotic integrity," *Cancer Research*, vol. 65, no. 3, pp. 850–860, 2005.

[68] D. Mori, Y. Yano, K. Toyo-Oka et al., "NDEL1 phosphorylation by aurora-A kinase is essential for centrosomal maturation, separation, and TACC3 recruitment," *Molecular and Cellular Biology*, vol. 27, no. 1, pp. 352–367, 2007.

Hindawi Publishing Corporation
Advances in Virology
Volume 2011, Article ID 432837, 10 pages
doi:10.1155/2011/432837

Review Article

XMRV Discovery and Prostate Cancer-Related Research

David E. Kang,[1,2] Michael C. Lee,[1,2] Jaydip Das Gupta,[2] Eric A. Klein,[1,2,3] and Robert H. Silverman[2,3]

[1] *Glickman Urological and Kidney Institute, Cleveland Clinic, 9500 Euclid Avenue, Cleveland, OH 44195, USA*
[2] *Department of Cancer Biology, Lerner Research Institute, Cleveland Clinic, 9500 Euclid Avenue, Cleveland, OH 44195, USA*
[3] *Taussig Cancer Center, Cleveland Clinic, 9500 Euclid Avenue, Cleveland, OH 44195, USA*

Correspondence should be addressed to Robert H. Silverman, silverr@ccf.org

Received 28 March 2011; Accepted 25 May 2011

Academic Editor: Arifa S. Khan

Xenotropic murine leukemia virus-related virus (XMRV) was first reported in 2006 in a study of human prostate cancer patients with genetic variants of the antiviral enzyme, RNase L. Subsequent investigations in North America, Europe, Asia, and Africa have either observed or failed to detect XMRV in patients (prostate cancer, chronic fatigue syndrome-myalgic encephalomyelitis (CFS-ME), and immunosuppressed with respiratory tract infections) or normal, healthy, control individuals. The principal confounding factors are the near ubiquitous presence of mouse-derived reagents, antibodies and cells, and often XMRV itself, in laboratories. XMRV infects and replicates well in many human cell lines, but especially in certain prostate cancer cell lines. XMRV also traffics to prostate in a nonhuman primate model of infection. Here, we will review the discovery of XMRV and then focus on prostate cancer-related research involving this intriguing virus.

1. Introduction

The retrovirus, xenotropic murine leukemia virus-related virus (XMRV), has generated both interest and debate within the scientific community and also among physicians, patients, and those concerned with maintaining the safety of blood and tissue banks around the world (reviewed in [1–3]). Its discovery was based on the hypothesis that viral infections might contribute to hereditary prostate cancer [4]. Currently, seven types of viruses (HPV, EBV, HHV-8, HTLV-1, HBV, HCV, and MCV) are established etiologic agents of different types of human cancers [5, 6]. While there is also evidence for the presence of viral infections in prostate cancer, including BKV [7], HPV [8, 9], HCMV [10], and EBV [11], thus far there is no compelling evidence that links viral infections to this disease. However, family history is a risk factor for prostate cancer, and in 2002, a combined positional cloning and candidate gene approach mapped a hereditary prostate cancer susceptibility locus, *HPC1* at 1q24-25 [12, 13], to the gene encoding the antiviral protein, RNase L [14]. While several studies have described a link between *RNASEL* and hereditary prostate cancer [14–18], other studies have been unable to confirm the association [19–22]. RNase L is one of the principal antiviral proteins in innate immunity [23]. Type I interferons produced during viral infections induce the pathogen recognition receptors, OAS1 to 3, which produce 2′,5′-oligo(rA) from ATP in response to viral double-stranded RNA. RNase L is present in most mammalian cell types and is activated upon binding to 2′,5′-oligo(rA), thus blocking viral infections by means of RNA degradation [24]. Many different types of viruses are susceptible, in particular viruses with single-stranded RNA genomes, including the retrovirus HIV-1 [25]. The mapping of *HPC1* to *RNASEL* and the invention of a global viral DNA microarray (aka virochip) provided the impetus and means for renewing the search for viruses in prostate cancer [26].

The realization that an HPC gene encoded an antiviral protein further suggested the possible involvement of viral infections in prostate cancer. To test this hypothesis, men with localized prostate cancer were genotyped for the R462Q (1385nt G→A) variant of *RNASEL*. A prior study showed that when homozygous this variant doubled the risk of prostate cancer and was implicated in up to 13% of cases [27]. The RNase L Q variant also has about 3-fold reduced

enzymatic activity compared with the wildtype R variant [27, 28]. Following radical prostatectomy, RNA isolated from prostate tumors was converted to labeled cDNA and used to screen for evidence of viral sequences by hybridization to virochips composed of the most conserved sequences of all known human, animal, plant, and bacterial viruses [4, 26, 29]. Because the array contained highly conserved sequences within viral nucleic acids, it can detect viruses not explicitly represented. These studies identified the presence of a *γ*retrovirus in 8 (40%) of 20 RNase L R462Q homozygous prostate cancer tissues, and in just 1 (1.5%) of 66 tissues that harbored at least one copy of the wildtype allele (Figure 1). Three XMRV genomes were completely sequenced and were found to share >98% nucleotide and >99% protein sequence identity. Partial sequences were obtained for another six XMRV strains. XMRV is more closely related to the xenotropic and polytropic than to the ecotropic murine retroviruses. XMRV is a canonical *γ*retrovirus, with *gag*, *pro-pol,* and *env* genes, and is not closely related to any endogenous human retroviral (HERV) elements (Figure 2). In addition, XMRV sequences are not present in any human genomic sequences that have been reported to date. A complete provirus clone for XMRV strain VP62 produced infectious virus in LNCaP or DU145 cells [30, 31]. XMRV is able to vigorously infect these and some other cell lines, in particular some prostate cancer cell lines [30, 32–34], allowing for basic virology studies in cell culture system to be conducted. A timeline of XMRV research shows that there was a lag prior to 2010 when a large increase in research papers on the subject appeared in peer-reviewed scientific journals (Figure 3).

2. Evidence for and against XMRV in Prostate Cancer

The possibility of laboratory contamination was carefully considered in the XMRV discovery paper in which several lines of evidence supported genuine human infections [4]. First, XMRV was detected using (DNase-treated) RNA directly isolated from fresh frozen, primary human prostate tumor tissues that were not placed in cell culture nor were these human samples exposed to any cultured cell products or cell culture reagents. Second, the extent of sequence variation between the different *gag* and *pol* sequences from different prostate cancer patients was greater than Taq polymerase error rates which range from 10^{-6} to 10^{-4} (see [66] and references therein). These finding suggested natural sequence diversity consistent with independent acquisition of XMRV infections by humans. Third, fluorescence in situ hybridization (FISH) identified XMRV nucleic acid in a small number of stromal cells in tumor-bearing prostate tissue. Fourth, a similar small number of Gag-positive stromal cells were detected in prostate tumor tissues using monoclonal antibody against spleen focus-forming virus Gag with an enhanced alkaline phosphatase red detection method. Fifth, no mouse GAPDH DNA sequences were detected in any of the radical prostatectomy samples providing evidence against contamination from any mouse-derived sources. Finally, XMRV was predominantly restricted to RNase L QQ prostate cancer cases. Therefore, both PCR based and non-PCR evidence supported genuine infection of humans.

Recently, however, the human origin of XMRV has been questioned based in part on the near sequence identity of XMRV strain VP62, isolated using human prostate cancer tissue, with XMRV present in a human prostate cancer cell line, 22Rv1 [33]. The 22Rv1 cells were derived in the 1990s at Case Western Reserve University from a human prostate cancer xenograft by serial passage in mice after castration-induced regression and relapse, raising the possibility that the virus in those cells originated from the mice rather than the patient [67]. A recent study shows that variation between XMRV sequences in the 22Rv1 cell lines exceeded that of XMRV sequences isolated from human specimens, leading the authors to propose that XMRV might not be an authentic human pathogen [59]. In addition, a recent study concludes that the XMRV in 22Rv1 cells originated from two MLV genomes present in the mice used to passage the xenografts ([45], 18th Conference on Retroviruses and Opportunistic Infections).

Additional evidence in favor of genuine infections, including some of the same prostate cancer patients as in the discovery study [4], was provided by XMRV integration site mapping experiments [30, 35]. DNA isolated from human prostate tissues in a PCR-amplicon-free clean room were sent to UCLA and used to precisely map and sequence XMRV integrations sites in nine separate prostate cancer patients [30, 35]. In these prostate cancer tissues, there was a greater tendency for XMRV to integrate near cancer-related genes, microRNA genes, common fragile sites, and cancer breakpoints in comparison to XMRV integration sites in DU145 prostate cancer cells infected in the lab [35]. Those results suggested an *in vivo* selection process for XMRV integrations in certain genes. The viral integration sites in human prostate DNA started precisely after the end of the right-side LTR repeat (5′-· · · CTTTCA-3′) demonstrating correct integration had occurred and ruling out artifactual fusion. These experiments also effectively ruled out direct mouse DNA contamination as a source of the XMRV sequences because LTR sequences were fused to human, not mouse, DNA. However, two of the integration sites are identical to XMRV integration sites obtained with XMRV-infected DU145 cells used in the same studies [35, 68, 69]. Therefore, it remains to be confirmed whether these two sites, and the other 12 XMRV integration sites, originated from the patients or from cells infected in the laboratory.

Three independent studies supported XMRV infections of prostate cancer patients at prevalence rates in the range of about 10 to 28%. A study from the University of Utah and Columbia University produced evidence for XMRV infection of prostatic malignant epithelial cells *in vivo* [31]. Immunohistochemistry (IHC) performed on 233 prostate cancer specimens and 101 controls with benign prostatic hyperplasia showed protein staining for XMRV in 23% of cancer cases and 4% of controls. IHC was performed using a polyclonal antibody raised in rabbit against whole virus XMRV (produced in 293T cells—a human kidney epithelial cell line). The presence of antibodies against host-derived

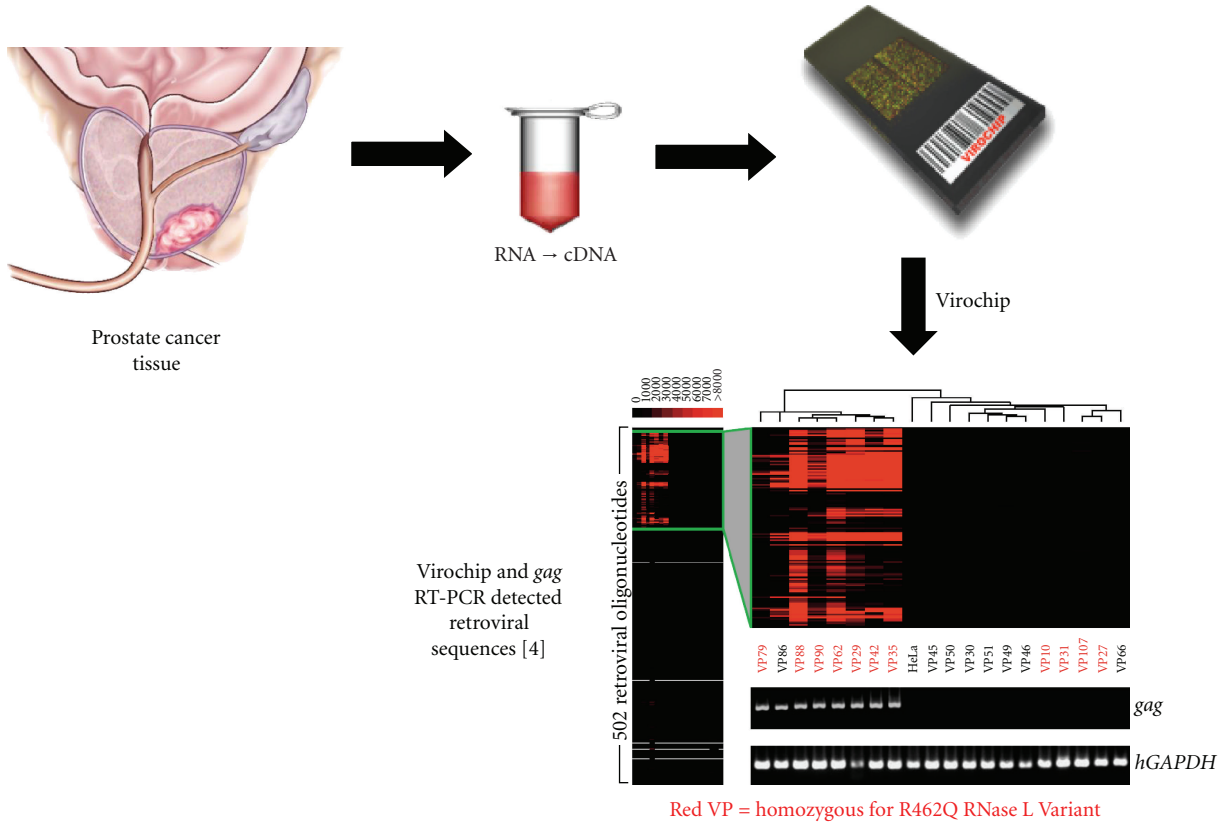


Figure 1: XMRV discovery in prostate cancer study [4]. Human prostate cancer tissues were collected in the operating room at the Cleveland Clinic and used to isolate RNA at either the Cleveland Clinic or at UCSF. At UCSF, RNA was used to synthesize labeled cDNA, virochips were probed, RT-PCR was performed for *gag* sequences, and XMRV cDNAs were sequenced. The RNase L genotypes were determined at the Cleveland Clinic (also the site of the IHC and FISH experiments, not shown). A hybridization pattern typical of a γretrovirus was obtained almost exclusively from patients with the RNase L QQ genotype (red bands and VP codes for QQ patients). RNase L RQ and RR genotypes are shown in black VP codes.

proteins among the anti-XMRV antibodies is a limitation of this approach and raises questions about whether the signals detected originated from XMRV infections. Quantitative PCR data from the same study detected XMRV DNA in 6.2% of prostate cancer and 2.0% of control specimens, much lower percentages than through IHC. XMRV associated with higher Gleason Index of prostate cancer but there was no correlation with the R462Q RNase L variant [31]. This report suggested that XMRV is a possible etiologic agent for prostate cancer, and not just a passenger virus.

An investigation at Emory University confirmed the presence of XMRV in men with prostate cancer by utilizing three methods, a novel serum-based assay for neutralizing antibodies against XMRV, nested PCR for *env* sequences, and FISH [53]. The serologic assay detected neutralizing antibodies in 11 of 40 prostate cancer cases (27.5%). Among 20 *RNASEL* QQ patients, 8 (40%) had neutralizing antibody against XMRV, in agreement with the original report of an association of XMRV infection with the QQ genotype [4]. FISH showed XMRV infection in 5–8% of stromal cells of positive cases, and none in epithelial cells [53]. All three methods were in agreement for 5 XMRV positive cases and 2 XMRV negative cases.

Most recently, a study at Baylor University detected XMRV in 32 of 144 (22%) men from the southern US with prostate cancer using a nested PCR assay for the *env* gene [54]. Patients were more likely to score positive for the presence of XMRV in both tumor and normal tissue than in either type of tissue alone. However, there was no correlation between the presence of XMRV and either the RNase L genotype or clinical parameters of disease. The presence of XMRV in normal tissues suggested that infection might precede prostate cancer.

In support of the presence of XMRV in some prostate cancer patients, a follow-up study at the Cleveland Clinic detected XMRV RNA by nested and quantitative RT-PCR of *env* RNA in expressed prostate secretions of 4 of 32 unselected prostate cancer cases [70]. These findings suggested that XMRV might be present in human semen. However, another study using nested RT-PCR of XMRV *gag* RNA failed to detect XMRV in seminal plasma from HIV-1-infected men in the Netherlands, although some of these subjects were using antiretroviral drugs [71].

Several research efforts have either failed to detect XMRV at all, or demonstrated very low prevalence of infection. In the first of several European studies, an investigation in

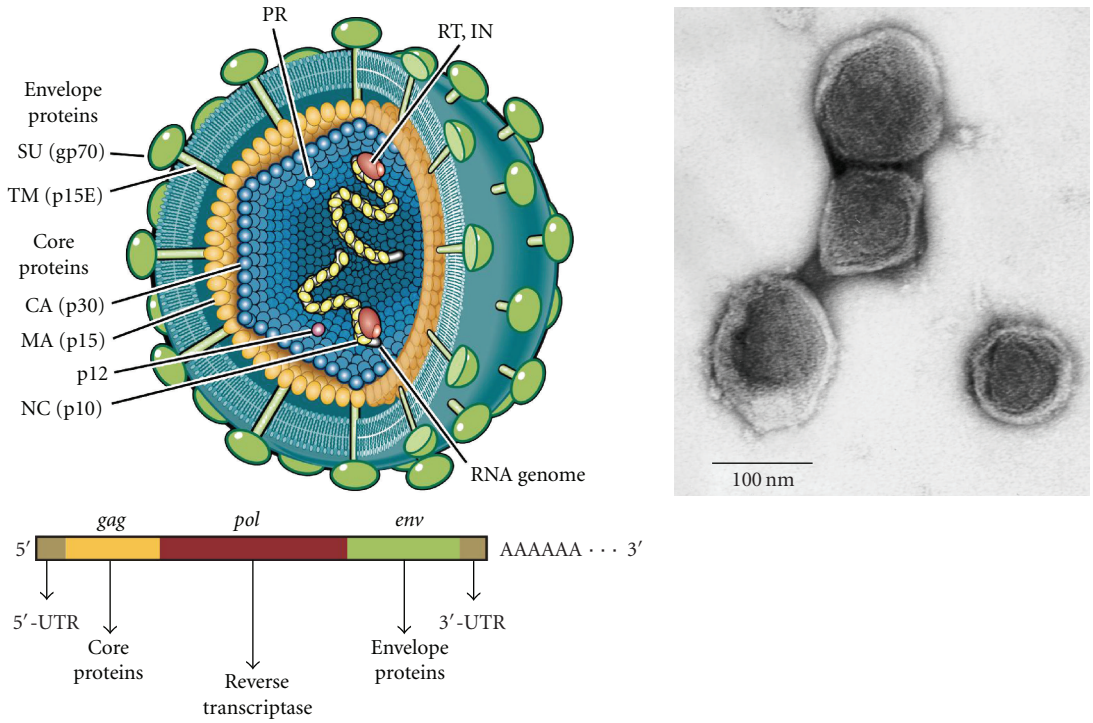


Figure 2: XMRV structure and morphology. (a) Structure of xenotropic murine leukemia virus-related virus showing viral core proteins from the *gag* gene (matrix (MA), capsid (CA), (IN), and nucleocapsid (NC) and p12); from *pol* (protease (PR), reverse transcriptase (RT), and integrase (IN)) and the envelope proteins (surface subunit (SU) and transmembrane subunit (TM) from *env*). Viral particles contain a lipid bilayer envelope and two RNA genomes. (b) Transmission electron microscope image of XMRV (courtesy of Dr. John Hackett, Jr., Abbott Diagnostics, Abbott Park, IL).

Hamburg, Germany found XMRV RNA by nested RT-PCR in only one of 87 cases in nonfamilial prostate cancer and one of 70 samples from a control population of men with benign prostatic hyperplasia [36]. Both patients contained at least one copy of the wildtype R462 RNase L allele. A study from Berlin failed to detect XMRV in 589 prostate cancer tissues and 146 prostate cancer serum samples utilizing nested PCR for *gag* DNA, RT-PCR for *gag* RNA, and serology assays for antibodies against XMRV Gag and Env. *RNASEL* status was examined in 76 patients in which 12.9% were QQ [42]. Further studies from Europe demonstrated similar negative results. Using PCR, a study of prostate cancer patients in Ireland found no evidence of XMRV DNA by PCR in 139 peripheral blood mononuclear cell (PBMC) samples nor in prostate tissues of seven *RNASEL* QQ prostate cancer cases and two RQ cases, although the QQ genotype was associated with more aggressive disease [72]. A Dutch study of tissue specimens for 74 sporadic prostate cancer patients showed low detection (1 in 600 to 7,000 cells) in only three cases (4%) using RT-PCR for XMRV *integrase* sequences [73]. An international collaborative study centered in the UK utilized nested PCR with *gag* leader primers on DNA extracted from formalin-fixed and paraffin-embedded (FFPE) prostate cancer tissues from the UK, Thailand, and Korea [60]. XMRV-like sequences were detected in 14/292 UK prostate cancers, 5/139 Korean samples, and 2/6 specimens from Thailand. However, upon sequencing, some amplified DNA fragments contained the 24 nt deletion upstream of the *gag* ATG start codon while other amplicons more closely resembled polytropic MLV. Because these results suggested contamination with mouse DNA, a single PCR assay for intracisternal A-type particle (IAP) LTR sequences and a TaqMan qPCR assay for mouse mitochondrial cytochrome oxidase, *cox2*, sequences were performed. These assays are highly sensitive due to large numbers of copies per mouse cell. The presence of the PCR products using XMRV *gag* leader primers in the human DNA samples was completely concordant with IAP sequences, the more sensitive of the two assays for mouse DNA contamination. Therefore, all "XMRV positive" samples were also contaminated with mouse DNA contamination.

Within North America, negative results have also been reported. In Mexico, XMRV RNA was assayed by nested RT-PCR with only a single positive out of 75 controls with an RNase L RR genotype, and no positives among 55 prostate cancer patients, none of which were QQ genotype [9]. An investigation of different bacteria and viruses in prostate cancer found no XMRV DNA in 200 patients using nested PCR to *gag* [74]. More recently a report that included some of the same authors published an absence of XMRV in over 800 specimens using both RT-PCR and IHC [55]. A duplex PCR assay was used on DNA from 161 prostatic adenocarcinomas in which XMRV *gag* sequences were coamplified with a host gene, but no XMRV was

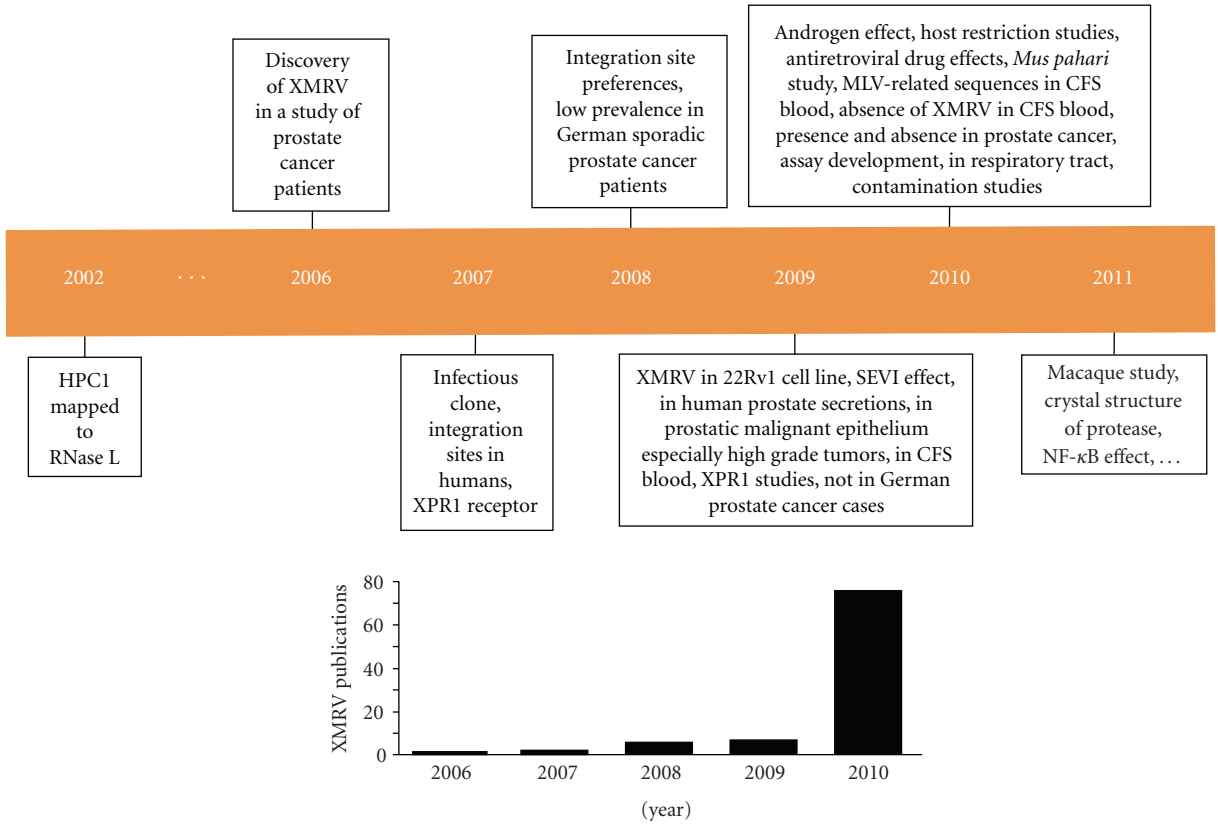


Figure 3: Timeline of XMRV research. Highlights of XMRV studies are shown, including many of the investigations discussed in this review. The mapping of *HPC1* to *RNASEL* was reported in 2002 [14] which led to the discovery of XMRV in 2006 using virochip technology [4]. In 2007, the first infectious clone of was constructed by fusing two overlapping cDNA from prostate cancer patient VP62 [30]. In addition, XPR1 was identified as the receptor for XMRV and the first integration sites in humans were reported [30]. In 2008, additional integration sites were mapped using human prostate cancer tissues [35]. A very low prevalence of XMRV was reported in sporadic prostate cancer patients in Germany [36]. In 2009, XMRV was identified in the human prostate cancer cell line 22Rv1, which had been repeatedly implanted and grown in mice [33]. In addition, a report of XMRV in prostatic malignant epithelium that correlated with tumor aggressiveness appeared [31]. The same year, a study using multiple methods of detection, including PCR, a serology assay for Env and isolation of live virus, showed XMRV in blood of CFS-ME patients, with much lower rates in healthy controls [37]. Studies into XPR1 function and specificity were reported between 2008 and 2010 [34, 38–41], including a study showing that whereas most laboratory strains of mice were resistant to infections, wild mice were susceptible [39]. A study from Germany that used PCR and antibody detection found no evidence of XMRV in prostate cancer [42]. In 2010, the androgen stimulatory effect on XMRV transcription and replication was reported [32, 43]. Host restriction factors, such as APOBEC3G and tetherin, were found to be active against XMRV [44–48]. Antiretroviral drugs were screened and some found to potently inhibit XMRV replication in cell culture [48–50]. The Asian mouse, *Mus pahari*, was exploited for studies on *in vivo* infection [50]. MLV-related sequences were found to associate with CFS-ME [51]. Meanwhile, several other studies, based on PCR and serology, failed to detect XMRV in CFS-ME (e.g., [52]). Two studies confirmed XMRV infections of prostate cancer patients [53, 54] while other studies failed to detect XMRV in prostate cancer patients in the US [55, 56]. Several assays for the detection of XMRV, including a high-throughput automated assay for antibodies against XMRV proteins, were reported [57]. XMRV was reported at a prevalence of almost 10% in immunosuppressed patients with respiratory tract infections in Germany [58]. Papers were published on laboratory contamination with mouse DNA that confounded the search for XMRV in humans [56, 59–62]. In early 2011, a study on XMRV in a non-human primate model showed wide-spread, persistent infection, including the prostate [63]. The crystal structure of the XMRV protease was published [64]. Finally, stimulation of XMRV transcription by proinflammatory cytokines through an NF-κB element in the LTR appeared [65].

detected. The assay was capable of detecting DNA from a single XMRV infected cell, 22Rv1, in the presence of a large excess of human DNA. In addition, 596 prostate cancers and 452 benign prostate tissue specimens were screened by IHC and all were negative. Recently, prostate tissue DNA from US patients with intermediate- to advanced-stage prostate cancer were tested by PCR assays for XMRV and MLV variants [75]. In three of 162 cases (1.9%), XMRV DNA was detected and sequenced. These samples were negative for mouse mitochondrial DNA using a highly sensitive assay, ruling out contamination from mouse DNA. There was no association with the RNase L QQ variant, plasma was negative for viral RNA by RT-PCR and all 162 patients were negative by a Western blot assay for antibody. The authors concluded that there was no association of XMRV or MLV variants with prostate cancer. However, they also concluded that there was a distinctive XMRV strain in 3 cases, demonstrating a broader diversity in this family of

viruses while supporting the case for human infections. A recent study of 110 prostate cancer cases and 40 benign or normal prostate tissues from the Midwestern US concluded no association with XMRV [56]. Although there were 6 PCR-positive cases for MLV sequences (5 prostate cancer and 1 nonprostate cancer) all of these were positive for mouse mitochondrial DNA suggesting contamination. There were sporadic IHC-positive prostate tissues using anti-XMRV antibody [31] but not with an anti-MLV antibody, and none of the serum samples produced strong neutralization of XMRV infections.

While the reasons for these disparate results have yet to be fully resolved, there are several possibilities. For instance, XMRV may be present at extremely low levels *in vivo* and therefore the virus could be missed. Because a single provirus can be transcribed into large numbers of RNA transcripts, detection of RNA may be more sensitive than that of DNA. Differences in geographical distribution of the virus, patient selection criteria, and methodology (e.g., PCR for *env* versus *gag* and tissue processing—e.g., fresh-frozen tissue versus formalin-fixed and paraffin-embedded tissues) are other variables. In some instances positive findings could be tainted by laboratory or reagent contamination, as is the case for mouse nucleic acids in some preparations of Platinum Taq polymerase (Invitrogen) used in PCR [1, 61]. Three examples of laboratory contamination have recently been published, two in prostate cancer specimens already mentioned [56, 60], the other in CFS-ME [62]. Because of the close relationship of XMRV sequence to sequences in mouse genomes, both extraordinary measures to avoid cross-contamination and ultrasensitive methods for detection of mouse DNA and RNA are necessary.

3. The Xenotropic and Polytropic Retrovirus Receptor 1 (XPR1) in Human Prostatic Cells

XPR1 is the cell surface receptor and determinant of viral infectivity for XMRV, X-MLVs, and P-MLVs [30, 34, 38, 39, 76–78]. It is a 696 aa protein with eight putative transmembrane domains and four putative extracellular loops (ECL1–4) [76–78]. Despite a common receptor, XMRV has host range and receptor requirements that differ from mouse X/P-MLVs, suggesting adaptations in humans or in intermediate hosts. Residues K500 and T582 in XPR1 ECL3 and ECL4, provide equivalent receptors for X/P-MLV, but not in the case of XMRV [40]. In addition, mouse X-MLV is able to infect all mammals, but XMRV is unique in being restricted in gerbil and hamster cells [40]. There are at least six functionally distinct variants of the XPR1 receptor with varying abilities to support entry by X-MLVs and P-MLVs [41]. While it is unknown whether XMRV found in humans was transmitted directly from infected mice, direct transmission could be reflected in the geographical distribution of virus and/or receptor type in mice, as well as in the worldwide distribution of prostate cancer cases. Interestingly, the most permissive *Xpr1* receptor allele, $Xpr1^{sxv}$, is found in areas of high prostate cancer incidence such as the United States, while the most restrictive allele, $Xpr1^{m}$, is found in low tumor rate areas such as Japan and Eastern Europe [38].

XPR1 RNA was shown to be present in human prostate stromal fibroblasts but absent in prostatic epithelial and smooth muscle cell lines [79], consistent with some previous findings that XMRV viral antigens are present in prostatic stromal fibroblasts of prostate cancer patients [4, 53]. However, prostate cancer cells of epithelial origin express XPR1 and are susceptible to XMRV infection [34]. XMRV was able to infect, at low levels, cells that did not express detectable levels of XPR1 RNA, suggesting an alternative pathway of infection [79].

4. Enhancement of XMRV Infectivity by Fibrils of Prostatic Acid Phosphatase Fragments

Prostatic acid phosphatase is the predominant protein in human semen, and fragments of this protein form positively charged amyloid fibrils that significantly increase HIV-1 infectivity [80]. These fibrils, aka "semen enhancers of virus infection" (SEVI), capture virus particles and greatly increase viral attachment and entry via cell surface receptors by neutralizing negative-charge repulsion between the HIV-1 virion and the cell surface [81]. SEVI has also been shown to enhance XMRV infections via the XPR1 receptor in human prostate cancer cell line DU145 [70]. SEVI enhanced XMRV attachment and fusion while lowering the threshold for infectivity by up to 4,000-fold. XMRV infectivity was enhanced by SEVI in a wide range of different cell types, including primary prostatic epithelial and stromal cells [70]. XMRV infectivity in cell culture was similarly enhanced by human semen, and this was most pronounced at low viral doses. These results, and the presence of XMRV RNA in prostate secretions, suggest sexual transmission as a potential biological mechanism for viral spread, although confirmation by seroprevalence and other epidemiologic studies is required before such a conclusion can be made. However, XMRV infection of rhesus macaques by the IV route showed that the virus traffics to and infects prostate epithelium within 6 to 7 days of infection [63]. In addition, a separate study, reported in this issue by Sharma et al., demonstrates that XMRV infects the reproductive tracts of both male and female macaques further suggesting the possibility of sexual transmission [82].

5. Host Restriction of XMRV in Prostate Cancer Cell Lines

Many host restriction factors are IFN regulated and collectively contribute to the IFN-induced antiviral state [83]. For example, IFNs induce OAS proteins that produce the 2′–5′-oligo(rA) activators of RNase L. As a result, RNase L suppresses replication of a wide range of viruses in cells exposed to IFN [24]. Sustained activation of RNase L also drives cells into apoptosis, a potential antitumor cell as well as an antiviral mechanism [84, 85]. Therefore, *RNASEL* mutations could contribute to prostate cancer by allowing clonal expansion of mutant cells that have escaped apoptosis and/or by allowing persistent infection by oncogenic viruses. Accordingly, reduction in RNase L levels by RNAi decreased the IFN antiviral effect against XMRV in DU145 cells [30].

However, in another study decreasing levels of RNase L using an RNAi approach did not enhance XMRV replication in 293T cells [32].

The human APOBEC3 family of cytidine deaminases includes seven members (A3A to H) encoded on chromosome 22 as a gene cluster [86]. A3G causes cytidine deamination in viral minus strand DNA causing G→A hypermutation in the coding strand thus potently inhibiting infectivity and spread of HIV-1 lacking Vif protein [87, 88]. XMRV lacks an inhibitor such as Vif and is highly susceptible to inhibition by A3G [44–47]. Accordingly, there was characteristic G-to-A hypermutation of XMRV DNA in T cell lines H9 and CEM that express A3G and A3F, but low levels of such mutations in prostate cancer cell lines, LNCaP, DU145, and 22Rv1, that lack A3G [45]. Primary prostatic stromal fibroblasts varied in expression of A3G mRNA from undetectable to moderate levels [46]. These findings suggest that prostate cancer cells and stromal fibroblast might provide a favorable environment for XMRV infection and replication *in vivo*.

Groom et al. investigated the effects of the murine protein Fv1 and the TRIM5α family of proteins [44]. XMRV was restricted by $Fv1^n$ and $Fv1^b$ but not restricted by any of 13 TRIM5 proteins tested. However, XMRV was highly susceptible to inhibition by the IFN-inducible protein, tetherin, that links viruses to the plasma membrane during budding [89].

6. Androgen Regulation of XMRV

Transcriptional control of the XMRV genome is mediated by *cis*-acting elements in the 5′-LTR U3 region. This 390-nucleotide segment contains the promoter and enhancers, as well as two glucocorticoid response elements (GRE). Other examples of GREs respond to glucocorticoids, mineralocorticoids, progesterone, and androgens. Furthermore, tropism studies of cultured cells suggest a role for the androgen receptor in promoting XMRV replication [30, 32, 34, 43]. XMRV was readily able to spread and replicate in androgen receptor positive LNCaP cells, but not in various other cell lines that lacked androgen receptor [32]. Dihydrotestosterone treatment of LNCaP cells caused a twofold and threefold increase of XMRV transcription and replication, respectively [43]. Conversely, the androgen inhibitors, casodex and flutamide, inhibited XMRV replication by up to threefold, which suggests that androgen ablation therapy used in prostate cancer treatment could inhibit viral growth [43]. A point mutation in one of the XMRV GREs led to impaired androgen regulation of XMRV transcription and replication [43]. Enhancer elements in the XMRV LTR could impart androgen regulation to integrated host genes, thus potentially contributing to oncogenesis.

7. Conclusions

There are a number of potential mechanisms by which a retrovirus could cause prostate cancer. Retroviruses generally transform cells by insertional activation of an oncogene, transduction of a host-derived oncogene, or oncogenesis by a viral protein (e.g., the JSRV Env protein) [90, 91]. γretroviruses, which lack a host-derived oncogene, typically cause cancer by insertion of the LTR near a cellular proto-oncogene leading to its activation. One can, however, envision possible alternative oncogenic mechanisms. For instance, viral infection in stromal cells might alter the microenvironment thus indirectly promoting neoplastic transformation. Infected stromal cells might induce cytokines, chemokines, or growth factors, creating a microenvironment conducive to tumorigenesis [92, 93]. Uncoordinated integration of viral DNA ends is another potential mechanism through which retroviruses may induce genomic alterations. However, it was recently shown that XMRV integration proceeds with high fidelity and involves a coordinated joining of the two viral DNA termini in the host genome flanked by a 4 bp direct repeat of host DNA [68].

XMRV does not have direct transforming activity in standard focus formation assays in fibroblast and epithelial cell lines [94, 95]. However, XMRV did rarely induce transformation of a rat fibroblast line, suggesting an indirect mode of action. Most likely, in order for XMRV to contribute to tumorigenesis through this mechanism, active viral replication with multiple integration events would be required until integration occurred in a cellular oncogene [94].

Claims of novel human retroviruses have often been met with considerable skepticism and resistance, earning the moniker human "rumor viruses" and XMRV is certainly no exception. While both PCR and non-PCR based evidence from several different laboratories collectively provide support for infections of some prostate cancer patients with XMRV, or similar viruses, an extreme level of caution is required to avoid laboratory contamination. Finally, only hypothesis-driven research that directly tests for infection and modes of pathogenesis for this virus can answer questions about its importance in disease.

Acknowledgments

These studies were supported by Grants from NIH (NCI) CA103943, US Department of Defense W81XWH-07-1-338, Mal and Lea Bank, the Charlotte Geyer Foundation, Abbott Laboratories, and the Milton and Tamar Maltz Family Foundation.

References

[1] R. H. Silverman, C. Nguyen, C. J. Weight, and E. A. Klein, "The human retrovirus XMRV in prostate cancer and chronic fatigue syndrome," *Nature Reviews Urology*, vol. 7, no. 7, pp. 392–402, 2010.

[2] J. P. Stoye, R. H. Silverman, C. A. Boucher, and S. F. J. Le Grice, "The xenotropic murine leukemia virus-related retrovirus debate continues at first international workshop," *Retrovirology*, vol. 7, article 113, 2010.

[3] H. G. Klein, R. Y. Dodd, F. B. Hollinger et al., "Xenotropic murine leukemia virus-related virus (XMRV) and blood transfusion: report of the AABB interorganizational XMRV task force," *Transfusion*, vol. 51, no. 3, pp. 654–661, 2011.

[4] A. Urisman, R. J. Molinaro, N. Fischer et al., "Identification of a novel gammaretrovirus in prostate tumors of patients

homozygous for R462Q *RNASEL* variant," *PLoS Pathogens*, vol. 2, no. 3, pp. 0211–0225, 2006.

[5] H. Fan, "A new human retrovirus associated with prostate cancer," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 5, pp. 1449–1450, 2007.

[6] P. S. Moore and Y. Chang, "Why do viruses cause cancer? Highlights of the first century of human tumour virology," *Nature Reviews Cancer*, vol. 10, no. 12, pp. 878–889, 2010.

[7] D. Das, R. B. Shah, and M. J. Imperiale, "Detection and expression of human BK virus sequences in neoplastic prostate tissues," *Oncogene*, vol. 23, no. 42, pp. 7031–7046, 2004.

[8] A. Zambrano, M. Kalantari, A. Simoneau, J. L. Jensen, and L. P. Villarreal, "Detection of human polyomaviruses and papillomaviruses in prostatictissue reveals the prostate as a habitat for multipleviral infections," *Prostate*, vol. 53, no. 4, pp. 263–276, 2002.

[9] M. L. Martinez-Fierro, R. J. Leach, L. S. Gomez-Guerra et al., "Identification of viral infections in the prostate and evaluation of their association with cancer," *BMC Cancer*, vol. 10, article 326, 2010.

[10] M. Samanta, L. Harkins, K. Klemm, W. J. Britt, and C. S. Cobbs, "High prevalence of human cytomegalovirus in prostatic intraepithelial neoplasia and prostatic carcinoma," *Journal of Urology*, vol. 170, no. 3, pp. 998–1002, 2003.

[11] S. Grinstein, M. V. Preciado, P. Gattuso et al., "Demonstration of Epstein-Barr virus in carcinomas of various sites," *Cancer Research*, vol. 62, no. 17, pp. 4876–4878, 2002.

[12] J. R. Smith, D. Freije, J. D. Carpten et al., "Major susceptibility locus for prostate cancer on chromosome 1 suggested by a genome-wide search," *Science*, vol. 274, no. 5291, pp. 1371–1374, 1996.

[13] J. D. Carpten, I. Makalowska, C. M. Robbins et al., "A 6-Mb high-resolution physical and transcription map encompassing the hereditary prostate cancer 1 (HPC1) region," *Genomics*, vol. 64, no. 1, pp. 1–14, 2000.

[14] J. Carpten, N. Nupponen, S. Isaacs et al., "Germline mutations in the ribonuclease L gene in families showing linkage with HPC1," *Nature Genetics*, vol. 30, no. 2, pp. 181–184, 2002.

[15] I. Agalliu, S. M. Leanza, L. Smith et al., "Contribution of HPC1 (*RNASEL*) and HPCX variants to prostate cancer in a founder population," *Prostate*, vol. 70, no. 15, pp. 1716–1727, 2010.

[16] A. Rökman, T. Ikonen, E. H. Seppälä et al., "Germline alterations of the *RNASEL* gene, a candidate HPC1 gene at 1q25, in patients and families with prostate cancer," *American Journal of Human Genetics*, vol. 70, no. 5, pp. 1299–1304, 2002.

[17] H. Rennert, D. Bercovich, A. Hubert et al., "A novel founder mutation in the *RNASEL* gene, 471delAAAG, is associated with prostate cancer in ashkenazi jews," *American Journal of Human Genetics*, vol. 71, no. 4, pp. 981–984, 2002.

[18] M. D. Fesinmeyer, E. M. Kwon, R. Fu, E. A. Ostrander, and J. L. Stanford, "Genetic variation in *RNASEL* and risk for prostate cancer in a population-based case-control study," *Prostate*. In press.

[19] S. R. Downing, K. T. Hennessy, M. Abe, J. Manola, D. J. George, and P. W. Kantoff, "Mutations in ribonuclease L gene do not occur at a greater frequency in patients with familial prostate cancer compared with patients with sporadic prostate cancer," *Clinical Prostate Cancer*, vol. 2, no. 3, pp. 177–180, 2003.

[20] H. Li and B. C. Tai, "*RNASEL* gene polymorphisms and the risk of prostate cancer: a meta-analysis," *Clinical Cancer Research*, vol. 12, no. 19, pp. 5713–5719, 2006.

[21] C. Maier, J. Haeusler, K. Herkommer et al., "Mutation screening and association study of *RNASEL* as a prostate cancer susceptibility gene," *British Journal of Cancer*, vol. 92, no. 6, pp. 1159–1164, 2005.

[22] F. Wiklund, B. A. Jonsson, A. J. Brookes et al., "Genetic analysis of the *RNASEL* gene in hereditary, familial, and sporadic prostate cancer," *Clinical Cancer Research*, vol. 10, no. 21, pp. 7150–7156, 2004.

[23] A. Chakrabarti, B. K. Jha, and R. H. Silverman, "New insights into the role of RNase L in innate immunity," *Journal of Interferon and Cytokine Research*, vol. 31, no. 1, pp. 49–57, 2011.

[24] R. H. Silverman, "Viral encounters with 2′,5′-oligoadenylate synthetase and RNase L during the interferon antiviral response," *Journal of Virology*, vol. 81, no. 23, pp. 12720–12729, 2007.

[25] R. K. Maitra and R. H. Silverman, "Regulation of human immunodeficiency virus replication by 2′,5′- oligoadenylate-dependent RNase L," *Journal of Virology*, vol. 72, no. 2, pp. 1146–1152, 1998.

[26] D. Wang, L. Coscoy, M. Zylberberg et al., "Microarray-based detection and genotyping of viral pathogens," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 24, pp. 15687–15692, 2002.

[27] G. Casey, P. J. Neville, S. J. Plummer et al., "*RNASEL* Arg462Gln variant is implicated in up to 13% of prostate cancer cases," *Nature Genetics*, vol. 32, no. 4, pp. 581–583, 2002.

[28] Y. Xiang, Z. Wang, J. Murakami et al., "Effects of RNase L mutations associated with prostate cancer on apoptosis induced by 2′,2′-Oligoadenylates," *Cancer Research*, vol. 63, no. 20, pp. 6795–6801, 2003.

[29] D. Wang, A. Urisman, Y. T. Liu et al., "Viral discovery and sequence recovery using DNA microarrays," *PLoS Biology*, vol. 1, no. 2, article e2, 2003.

[30] B. Dong, S. Kim, S. Hong et al., "An infectious retrovirus susceptible to an IFN antiviral pathway from human prostate tumors," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 5, pp. 1655–1660, 2007.

[31] R. Schlaberg, D. J. Choe, K. R. Brown, H. M. Thaker, and I. R. Singh, "XMRV is present in malignant prostatic epithelium and is associated with prostate cancer, especially high-grade tumors," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 38, pp. 16351–16356, 2009.

[32] J. J. Rodriguez and S. P. Goff, "Xenotropic murine leukemia virus-related virus establishes an efficient spreading infection and exhibits enhanced transcriptional activity in prostate carcinoma cells," *Journal of Virology*, vol. 84, no. 5, pp. 2556–2562, 2010.

[33] E. C. Knouf, M. J. Metzger, P. S. Mitchell et al., "Multiple integrated copies and high-level production of the human retrovirus XMRV (Xenotropic Murine leukemia virus-Related Virus) from 22Rv1 prostate carcinoma cells," *Journal of Virology*, vol. 83, no. 14, pp. 7353–7356, 2009.

[34] K. Stieler, C. Schulz, M. Lavanya, M. Aepfelbacher, C. Stocking, and N. Fischer, "Host range and cellular tropism of the human exogenous gammaretrovirus XMRV," *Virology*, vol. 399, no. 1, pp. 23–30, 2010.

[35] S. Kim, N. Kim, B. Dong et al., "Integration site preference of xenotropic murine leukemia virus-related virus, a new human retrovirus associated with prostate cancer," *Journal of Virology*, vol. 82, no. 20, pp. 9964–9977, 2008.

[36] N. Fischer, O. Hellwinkel, C. Schulz et al., "Prevalence of human gammaretrovirus XMRV in sporadic prostate cancer," *Journal of Clinical Virology*, vol. 43, no. 3, pp. 277–283, 2008.

[37] V. C. Lombardi, F. W. Ruscetti, J. D. Gupta et al., "Detection of an infectious retrovirus, XMRV, in blood cells of patients with chronic fatigue syndrome," *Science*, vol. 326, no. 5952, pp. 585–589, 2009.

[38] C. A. Kozak, "The mouse "xenotropic" gammaretroviruses and their XPR1 receptor," *Retrovirology*, vol. 7, article 101, 2010.

[39] S. Baliji, Q. Liu, and C. A. Kozak, "Common inbred strains of the laboratory mouse that are susceptible to infection by mouse xenotropic gammaretroviruses and the human-derived retrovirus XMRV," *Journal of Virology*, vol. 84, no. 24, pp. 12841–12849, 2010.

[40] Y. Yan, Q. Liu, K. Wollenberg, C. Martin, A. Buckler-White, and C. A. Kozak, "Evolution of functional and sequence variants of the mammalian XPR1 receptor for mouse xenotropic gammaretroviruses and the human-derived retrovirus XMRV," *Journal of Virology*, vol. 84, no. 22, pp. 11970–11980, 2010.

[41] Y. Yan, Q. Liu, and C. A. Kozak, "Six host range variants of the xenotropic/polytropic gammaretroviruses define determinants for entry in the XPR1 cell surface receptor," *Retrovirology*, vol. 6, article 87, 2009.

[42] O. Hohn, H. Krause, P. Barbarotto et al., "Lack of evidence for xenotropic murine leukemia virus-related virus(XMRV) in German prostate cancer patients," *Retrovirology*, vol. 6, article 92, 2009.

[43] B. Dong and R. H. Silverman, "Androgen stimulates transcription and replication of xenotropic murine leukemia virus-related virus," *Journal of Virology*, vol. 84, no. 3, pp. 1648–1651, 2010.

[44] H. C. T. Groom, M. W. Yap, R. P. Galão, S. J. D. Neil, and K. N. Bishop, "Susceptibility of xenotropic murine leukemia virus-related virus (XMRV) to retroviral restriction factors," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 11, pp. 5166–5171, 2010.

[45] T. Paprotka, N. J. Venkatachari, C. Chaipan et al., "Inhibition of xenotropic murine leukemia virus-related virus by APOBEC3 proteins and antiviral drugs," *Journal of Virology*, vol. 84, no. 11, pp. 5719–5729, 2010.

[46] K. Stieler and N. Fischer, "Apobec 3G efficiently reduces infectivity of the human exogenous gammaretrovirus XMRV," *PLoS ONE*, vol. 5, no. 7, Article ID e11738, 2010.

[47] H. P. Bogerd, F. Zhang, P. D. Bieniasz, and B. R. Cullen, "Human APOBEC3 proteins can inhibit xenotropic murine leukemia virus-related virus infectivity," *Virology*, vol. 410, no. 1, pp. 234–239, 2011.

[48] R. A. Smith, G. S. Gottlieb, and A. D. Miller, "Susceptibility of the human retrovirus XMRV to antiretroviral inhibitors," *Retrovirology*, vol. 7, article 70, 2010.

[49] I. R. Singh, J. E. Gorzynski, D. Drobysheva, L. Bassit, and R. F. Schinazi, "Raltegravir is a potent inhibitor of XMRV, a virus implicated in prostate cancer and chronic fatigue syndrome," *PLoS ONE*, vol. 5, no. 4, Article ID e9948, 2010.

[50] T. Sakuma, J. M. Tonne, K. A. Squillace et al., "Early events in retrovirus XMRV infection of the wild-derived mouse Mus pahari," *Journal of Virology*, vol. 85, no. 3, pp. 1205–1213, 2011.

[51] S. C. Lo, N. Pripuzova, B. Li et al., "Detection of MLV-related virus gene sequences in blood of patients with chronic fatigue syndrome and healthy blood donors," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 36, pp. 15874–15879, 2010.

[52] O. Erlwein, S. Kaye, M. O. McClure et al., "Failure to detect the novel retrovirus XMRV in chronic fatigue syndrome," *PLoS ONE*, vol. 5, no. 1, Article ID e8519, 2010.

[53] R. S. Arnold, N. V. Makarova, A. O. Osunkoya et al., "XMRV infection in patients with prostate cancer: novel serologic assay and correlation With PCR and FISH," *Urology*, vol. 75, no. 4, pp. 755–761, 2010.

[54] B. P. Danielson, G. E. Ayala, and J. T. Kimata, "Detection of xenotropic murine leukemia virus-related virus in normal and tumor tissue of patients from the southern United States with prostate cancer is dependent on specific polymerase chain reaction conditions," *Journal of Infectious Diseases*, vol. 202, no. 10, pp. 1470–1477, 2010.

[55] A. L. Aloia, K. S. Sfanos, W. B. Isaacs et al., "XMRV: a new virus in prostate cancer?" *Cancer Research*, vol. 70, no. 24, pp. 10028–10033, 2010.

[56] T. Sakuma, S. Hué, K. A. Squillace et al., "No evidence of XMRV in prostate cancer cohorts in the Midwestern United States," *Retrovirology*, vol. 8, article 23, 2011.

[57] X. Qiu, P. Swanson, K. C. Luk et al., "Characterization of antibodies elicited by XMRV infection and development of immunoassays useful for epidemiologic studies," *Retrovirology*, vol. 7, article 68, 2010.

[58] N. Fischer, C. Schulz, K. Stieler et al., "Xenotropic murine leukemia virus-related gammaretro virus in respiratory tract," *Emerging Infectious Diseases*, vol. 16, no. 6, pp. 1000–1002, 2010.

[59] S. Hue, E. R. Gray, A. Gall et al., "Disease-associated XMRV sequences are consistent with laboratory contamination," *Retrovirology*, vol. 7, article 111, 2010.

[60] M. J. Robinson, O. W. Erlwein, S. Kaye et al., "Mouse DNA contamination in human tissue tested for XMRV," *Retrovirology*, vol. 7, 108, 2010.

[61] E. Sato, R. A. Furuta, and T. Miyazawa, "An endogenous murine leukemia viral genome contaminant in a commercial RT-PCR Kit is amplified using standard primers for XMRV," *Retrovirology*, vol. 7, article 110, 2010.

[62] B. Oakes, A. K. Tai, O. Cingoz et al., "Contamination of human DNA samples with mouse DNA can lead to false detection of XMRV-like sequences," *Retrovirology*, vol. 7, article 109, 2010.

[63] N. Onlamoon, J. D. Gupta, P. Sharma et al., "Infection, viral dissemination, and antibody responses of rhesus macaques exposed to the human gammaretrovirus XMRV," *Journal of Virology*, vol. 85, no. 9, pp. 4547–4557, 2011.

[64] M. Li, F. Dimaio, D. Zhou et al., "Crystal structure of XMRV protease differs from the structures of other retropepsins," *Nature Structural and Molecular Biology*, vol. 18, no. 2, pp. 227–229, 2011.

[65] S. Sakakibara, K. Sakakibara, and G. Tosato, "NF-κB activation stimulates transcription and replication of retrovirus XMRV in human B-lineage and prostate carcinoma cells," *Journal of Virology*, vol. 85, no. 7, pp. 3179–3186, 2011.

[66] M. A. Bracho, A. Moya, and E. Barrio, "Contribution of Taq polymerase-induced errors to the estimation of RNA virus diversity," *Journal of General Virology*, vol. 79, no. 12, pp. 2921–2928, 1998.

[67] R. M. Sramkoski, T. G. Pretlow, J. M. Giaconia et al., "A new human prostate carcinoma cell line, 22Rv1," *In Vitro Cellular and Developmental Biology*, vol. 35, no. 7, pp. 403–409, 1999.

[68] S. Kim, A. Rusmevichientong, B. Dong, R. Remenyi, R. H. Silverman, and S. A. Chow, "Fidelity of target site duplication and sequence preference during integration of Xenotropic murine leukemia virus-related virus," *PLoS ONE*, vol. 5, no. 4, Article ID e10255, 2010.

[69] J. A. Garson, P. Kellam, and G. J. Towers, "Analysis of XMRV integration sites from human prostate cancer tissues suggests

PCR contamination rather than genuine human infection," *Retrovirology*, vol. 8, article 13, 2011.

[70] S. Hong, E. A. Klein, J. Das Gupta et al., "Fibrils of prostatic acid phosphatase fragments boost infections with XMRV (Xenotropic Murine leukemia virus-Related Virus), a human retrovirus associated with prostate cancer," *Journal of Virology*, vol. 83, no. 14, pp. 6995–7003, 2009.

[71] M. Cornelissen, F. Zorgdrager, P. Blom et al., "Lack of detection of XMRV in seminal plasma from HIV-1 infected men in The Netherlands," *PLoS ONE*, vol. 5, no. 8, Article ID e12040, 2010.

[72] F. D'Arcy, R. Foley, A. Perry et al., "No evidence of XMRV in Irish prostate cancer patients with R462Q mutation.," *European Urology*, supplement 7, p. 271, 2008.

[73] G. W. Verhaegh, A. S. De Jong, F. P. Smit, S. A. Jannink, W. J.G. Melchers, and J. A. Schalken, "Prevalence of human xenotropic murine leukemia virus-related gammaretrovirus (XMRV) in dutch prostate cancer patients," *Prostate*, vol. 71, no. 4, pp. 415–420, 2011.

[74] K. S. Sfanos, J. Sauvageot, H. L. Fedor, J. D. Dick, A. M. De Marzo, and W. B. Isaacs, "A molecular analysis of prokaryotic and viral DNA sequences in prostate tissue from patients with prostate cancer indicates the presence of multiple and diverse microorganisms," *Prostate*, vol. 68, no. 3, pp. 306–320, 2008.

[75] W. M. Switzer, H. Jia, H. Zheng, S. Tang, and W. Heneine, "No association of xenotropic murine leukemia virus-related viruse with prostate cancer," *PLoS ONE*, vol. 6, article e19065, 2011.

[76] J. L. Battini, J. E. J. Rasko, and A. D. Miller, "A human cell-surface receptor for xenotropic and polytropic murine leukemia viruses: possible role in G protein-coupled signal transduction," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 4, pp. 1385–1390, 1999.

[77] C. S. Tailor, A. Nouri, C. G. Lee, C. Kozak, and D. Kabat, "Cloning and characterization of a cell surface receptor for xenotropic and polytropic marine leukemia viruses," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 3, pp. 927–932, 1999.

[78] Y. L. Yang, L. Guo, S. Xu et al., "Receptors for polytropic and xenotropic mouse leukaemia viruses encoded by a single gene at Rmc1," *Nature Genetics*, vol. 21, no. 2, pp. 216–219, 1999.

[79] S. Bhosle, S. Suppiah, R. Molinaro et al., "Evaluation of cellular determinants required for in vitro xenotropic murine leukemia virus-related virus entry into human prostate cancer and noncancerous cells," *Journal of Virology*, vol. 84, no. 13, pp. 6288–6296, 2010.

[80] J. Münch, E. Rücker, L. Ständker et al., "Semen-derived amyloid fibrils drastically enhance HIV infection," *Cell*, vol. 131, no. 6, pp. 1059–1071, 2007.

[81] N. R. Roan, J. Münch, N. Arhel et al., "The cationic properties of SEVI underlie its ability to enhance human immunodeficiency virus infection," *Journal of Virology*, vol. 83, no. 1, pp. 73–80, 2009.

[82] P. Sharma, K. A. Rogers, S. Suppiah et al., "Sexual transmission of XMRV: a potential infection route," *Advances in Virology*. In press.

[83] R. N. Douville and J. Hiscott, "The interface between the innate interferon response and expression of host retroviral restriction factors," *Cytokine*, 2010.

[84] A. Zhou, J. Paranjape, T. L. Brown et al., "Interferon action and apoptosis are defective in mice devoid of 2',5'-oligoadenylate-dependent RNase L," *EMBO Journal*, vol. 16, no. 21, pp. 6355–6363, 1997.

[85] J. C. Castelli, B. A. Hassel, K. A. Wood et al., "A study of the interferon antiviral mechanism: apoptosis activation by the 2-5A system," *Journal of Experimental Medicine*, vol. 186, no. 6, pp. 967–972, 1997.

[86] S. G. Conticello, C. J. F. Thomas, S. K. Petersen-Mahrt, and M. S. Neuberger, "Evolution of the AID/APOBEC family of polynucleotide (deoxy)cytidine deaminases," *Molecular Biology and Evolution*, vol. 22, no. 2, pp. 367–377, 2005.

[87] A. M. Sheehy, N. C. Gaddis, J. D. Choi, and M. H. Malim, "Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein," *Nature*, vol. 418, no. 6898, pp. 646–650, 2002.

[88] B. R. Cullen, "Role and mechanism of action of the APOBEC3 family of antiretroviral resistance factors," *Journal of Virology*, vol. 80, no. 3, pp. 1067–1076, 2006.

[89] S. J. D. Neil, T. Zang, and P. D. Bieniasz, "Tetherin inhibits retrovirus release and is antagonized by HIV-1 Vpu," *Nature*, vol. 451, no. 7177, pp. 425–430, 2008.

[90] N. Maeda, M. Palmarini, C. Murgia, and H. Fan, "Direct transformation of rodent fibroblasts by jaagsiekte sheep retrovirus DNA," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 8, pp. 4449–4454, 2001.

[91] S. K. Wootton, C. L. Halbert, and A. D. Miller, "Sheep retrovirus structural protein induces lung tumours," *Nature*, vol. 434, no. 7035, pp. 904–907, 2005.

[92] N. A. Bhowmick, E. G. Neilson, and H. L. Moses, "Stromal fibroblasts in cancer initiation and progression," *Nature*, vol. 432, no. 7015, pp. 332–337, 2004.

[93] T. D. Tlsty and P. W. Hein, "Know thy neighbor: stromal cells can contribute oncogenic signals," *Current Opinion in Genetics and Development*, vol. 11, no. 1, pp. 54–59, 2001.

[94] M. J. Metzger, C. J. Holguin, R. Mendoza, and A. D. Miller, "The prostate cancer-associated human retrovirus XMRV lacks direct transforming activity but can induce low rates of transformation in cultured cells," *Journal of Virology*, vol. 84, no. 4, pp. 1874–1880, 2010.

[95] M. J. Metzger and A. D. Miller, "Acutely transforming retrovirus expressing Nras generated from HT-1080 fibrosarcoma cells infected with the human retrovirus XMRV," *Journal of Virology*, vol. 84, no. 15, pp. 7908–7910, 2010.

Hindawi Publishing Corporation
Advances in Virology
Volume 2011, Article ID 281425, 5 pages
doi:10.1155/2011/281425

Review Article

Testing Strategies for Detection of Xenotropic Murine Leukemia Virus-Related Virus Infection

Shixing Tang and Indira K. Hewlett

Laboratory of Molecular Virology, CBER, Food and Drug Administration, Bethesda, MD 20892, USA

Correspondence should be addressed to Shixing Tang, shixing.tang@fda.hhs.gov

Received 10 March 2011; Revised 18 May 2011; Accepted 10 June 2011

Academic Editor: Myra McClure

Xenotropic murine leukemia virus-related virus (XMRV) is a newly identified gamma retrovirus and may be associated with prostate cancer- (PC) and chronic fatigue syndrome (CFS). Since its identification in 2006 and detection of polytropic murine lenkemia virus (MLV)-like sequences in CFS patients in 2010, several test methods including nucleic acid testing methods and serological assays have been developed for detection of XMRV and/or MLV-like sequences. However, these research assays have not yet been validated and evaluated due to the lack of well-characterized reference materials. Mouse DNA contamination should be carefully checked when testing human specimens in order to avoid false-positive detection of XMRV or MLV-like sequences.

1. Introduction

When XMRV was first identified in PC patients in 2006 [1], it did not get much public attention until a science paper published in 2009 reported its detection in a majority (67%) of CFS patients and about 4% of healthy Americans [2]. In this report, XMRV was shown to be infectious and could be isolated from peripheral mononuclear cells (PBMCs) and plasma, indicating that it is the first gamma retrovirus that infects humans and may be associated with human diseases [1, 2]. Furthermore, if confirmed, it was thought that millions of persons worldwide may harbor the new virus and thus pose a serious concern to public health and the safety of blood transfusion and organ transplantation. These findings greatly stimulated the interest of scientists in academia and government agencies to address both public health and scientific concerns about the newly identified retrovirus and its possible association with human diseases. However, the studies that followed the original publications have yielded conflicting findings and generated more controversy than consensus about XMRV detection and its potential disease association (see reviews [3–11]). In 2010, Lo et al. reported the detection of polytropic MLV-like sequences in 87% of CFS patients [12]. MLV-like sequences are different from, but very similar to, XMRV [12]. The detection of polytropic MLV in CFS patients suggested that XMRV may be only one of an apparent cluster of MLV-like viruses identified in patient specimens. In this paper, we describe and summarize the various testing methods and assays that have been employed for detection of XMRV and/or MLV-like virus infection in the studies that have been published until the present time.

2. XMRV Testing Methods

A variety of test methods have been employed to detect XMRV in cell culture studies and clinical specimens. Polymerase chain reaction (PCR) assay (nested and real-time PCR), transcription-mediated amplification assay (TMA), and fluorescence *in situ* hybridization (FISH) have been used for direct detection of viral sequences. Several serologic assays for detection of circulating antibodies against XMRV have been reported, including flow cytometry (FACS), Western blot (WB), chemiluminescence-based immunoassays and enzyme linked immunosorbent assay (ELISA), and so forth. Immunohistochemical staining (IHC) has been used for direct detection of viral proteins, while cell culture assays were used for isolation and detection of infectious virus.

2.1. Nucleic Acid Testing (NAT). Viral nucleic acid detection by reverse transcription- (RT-) based PCR or DNA PCR methods has been widely used for detection of XMRV and/or MLV-like sequences, but the results have generated controversial findings partly because of the differences in detection sensitivity and specificity [11, 13, 14]. Several issues need to be considered when using PCR to detect XMRV and/or MLV-like sequences. Mikovits et al. emphasized that the PCR template is critical for successful amplification and relatively high-detection rate of XMRV [15, 16]. They found that by using DNA extracted from inactivated PBMCs as template, only 7% and 21% of CFS patients were positive in single-round and nested PCR, respectively. By using viral RNA template from activated PBMC and cocultured cells and RT PCR, the detection rate was 72% and 89%, respectively [15, 16]. Using activated PBMC can significantly improve detection sensitivity because MLVs and other closely related retroviruses rely on mitosis to access the host cell chromosomes [17] and the copies of XMRV in activated cells are increased. Their results suggested that it may be more sensitive to detect viral RNA rather than proviral DNA, which was done in their original science paper [2]. The amount of DNA in the template is also critical. Danielson et al. found that XMRV was detected in 3.2% of the patients when 100–140 ng of prostate tissue DNA were used, compared with the positivity of 22.2% when 650 ng DNA were used [18]. However, Lo et al. reported that 87% of CFS patients were positive for MLV-like viral sequences when less than 50 ng of DNA per reaction and similar primers from XMRV were used [12]. The selection of PCR primers is another crucial factor that may affect detection of XMRV-specific sequences. The PCR primers previously claimed to be specific for XMRV have been found to be able to readily amplify MLV sequences from a variety of mice and some human cell lines [19–22], including polytropic MLV-like sequences identified by Lo et al. [12]. The typical 24 nucleotide deletion within the *gag* leader region of the XMRV genome is also found in some MLV sequences, endogenous retroviruses and in several mouse strains [19, 22]. In addition, Chow and Ikeda did not detect the 24-nt deletion in the *gag* leader region in their studies [14]. These results indicate that it may be difficult to design PCR primers that are exclusively specific for XMRV due to the high-sequence homology between XMRV and MLVs and even endogenous retroviral sequences. However, Schlaberg et al. reported a quantitative PCR for specific detection of XMRV by using primers from XMRV integrase gene, which are 100% conserved in 3 published XMRV sequences (VP35, VP42, and VP62) and share only 80–85% sequence identity with the most similar murine retroviruses [23]. They could consistently detect 50 copies of the XMRV proviral clone and 5 copies at 50% of hit rate [23]. Van Kuppeveld et al. also observed that both the real time PCR assay and the nested PCR assay could detect at least 10 copies of XMRV, indicating similar sensitivity [24]. Some studies found that XMRV could only be amplified by *gag* primers but not *env* or *pol* primers, or vice versa; or that detection sensitivity relies on primer sequences and locations. Danielson et al. reported that XMRV *gag* primers were at least 10-fold less sensitive than *env* primers; and *pol* primers tended to amplify a competing region from the human genome. They could not detect XMRV in patient tissue samples by nested RT-PCR with primers specific for *gag* and *pol* genes, regardless of whether 100 or 650 ng of DNA was used as template [18]. Stoye et al. reported that the XMRV *env* primers yielded the most positive results [14]. However, Lo et al. found *gag* primers were more sensitive than *env* primers when detecting MLV-like sequences [12]. Oakes et al. found that 53% (19/36) of the healthy volunteers and 1.8% (2/112) of the CFS patients yielded PCR products when using XMRV *gag* primers, but no positive amplification was observed when using qPCR with *pol* primers [20]. Switzer et al. [36] tested 162 PC patients and found that PCR products were obtained for *gag, pol,* and *env* from one patient, from *pol* and *env* from a second, and *pol* alone from a third case. Furthermore, PCR was not successful in all replicates on individual samples, indicating that multiple primer sets and repeats may be necessary for accurate detection of XMRV, possibly due to the very low viral titers in clinical samples [14]. The real reasons are not clear due to lack of consensus results. Although XMRV sequences appear to be highly conserved, variations have been consistently mentioned and may account for some of the negative results [5, 15, 25].

A major concern in regard to XMRV detection by PCR is false-positive results caused by contamination. Recently, four publications that appeared in the journal Retrovirology identified three potential sources of contamination in PCR-based studies of XMRV. Robinson and Oakes et al. reported independently that all XMRV-positive samples in their analysis were also positive for mouse DNA when assayed using a mouse mitochondrial DNA PCR or intracisternal A particle (IAP) assay [19, 20]. Hue et al. confirmed that 2.2% (5/411) of human cell lines screened were positive for MLVs [22]. Sato et al. found that commercial RT-PCR reagents were contaminated with MLV RNA [21]. The contamination was thought to originate from the hybridoma cell line from which the monoclonal antibody used in the polymerase reaction mixture to facilitate hot-start PCR was prepared. These results indicate that mouse DNA contamination is widespread and can confound XMRV detection in human samples. Furthermore, Hue et al. compared the published XMRV sequences with those from XMRV positive 22Rv1 cell, and found the genetic distance among 22Rv1-derived sequences exceeds that of XMRV sequences from patients, indicating that XMRV detected in patients may result from laboratory contamination rather than a true human infection [22]. Interestingly, 22Rv1 cell line was derived from a human prostate cancer xenograft (CWR22) that was serially passaged in nude mice in 1990s. Paprotka et al. recently reported that XMRV was the recombinant of two endogenous MLVs during passage of the CWR22 PC xenograft [26], suggesting that XMRV is a laboratory-derived virus and may have contaminated samples for more than a decade, but it may not infect people. These results clearly show that extraprecautions must be taken to avoid mouse DNA contamination and false-positive amplification from human cell lines that harbor xenotropic MLVs since they are

closely related to XMRV. PCR methods have been used to detect mouse mitochondrial DNA [12], cox2 DNA [20], and IAP [19, 20]. IAPs are endogenous transportable elements present at the level of about 1000 copies per mouse genome [27]. Robinson et al. found the mtDNA PCR was 100-fold less sensitive than that for IAP when testing in both McCoy cell and RAW 264.7 cell DNA [19], suggesting that IAP PCR may be more suitable for finding contamination of murine sequences.

Due to concerns about variations in nucleic acid-based methods that have been used to study XMRV, the National Heart, Lung, and Blood Institute (NHLBI) under the leadership of the Health and Human Services (HHS) sponsored an XMRV Blood Scientific Working Group (BSWG) to validate the testing assays that have been developed and used in different laboratories for CFS and blood donor testing [28]. Preliminary results of this working group indicated that most nucleic acid testing assays used in different labs were able to achieve similar levels of sensitivity and specificity based on the spiked XMRV panels [28]. However, when testing CFS samples, no consensus of results was observed. Therefore, it is still not clear if assay methodology alone could account for the large differences observed when testing clinical samples.

2.2. Serology. Antibody detection is considered as strong evidence of XMRV infection, in particular, in the absence of positive PCR results, or when the PCR results are not very reliable due to differences in assay sensitivity and specificity. High-throughput serologic assays that detect XMRV-specific antibodies would be of great value for determining the epidemiology of possible XMRV infection and for addressing the association of XMRV infection and human diseases if they exist at all. However, the nature and kinetics of the antibody response to XMRV infection have not been well-characterized; or it may be premature to discuss the antibody responses since XMRV infection of humans has yet to be proven.

Lombardi et al. first reported on specific immune responses to XMRV in CFS patients [2]. They used FACS to measure the antibody against XMRV by mixing patient plasma with a mouse B cell line expressing recombinant spleen focus-forming virus (SFFV) *env* protein. Because XMRV shares >90% overall nucleotide sequence identity with known MLVs, cross-reactivity between anti-MLV antibodies and XMRV proteins has been observed and 50% (9/18) of CFS patients were shown to be reactive [2]. Furthermore, anti-XMRV-positive plasma samples from CFS patients blocked the binding of anti-SFFV *env* antibody to SFFV *env* on the cell surface. They claimed that FACS was one of the most sensitive blood-based assays for detection of anti-XMRV *env* antibody in patient plasma and could detect 82% (47/57) of XMRV infection in CFS patients [15, 16]. Arnold et al. developed a single-round reporter gene based on neutralizing antibody assay and found that 27.5% (11/40) PC patients were anti-XMRV antibody positive [29]. In their system, anti-XMRV antibody was confirmed by blocking the binding and infection of XMRV-HIV pseudovirus expressing XMRV *env* protein with the reporter cell line JC53BL-13 that carries the firefly luciferase and β-galactosidase under the control of the HIV-1 LTR. The results were in complete concordance with both PCR and FISH for 7 patients in whom 3 assays were conducted [29]. However, Sabunciya et al. did not find differences in immunoreactivity between PC patients and controls by ELISA using XMRV *env* or *gag* antigen to capture anti-XMRV antibodies [30]. A similar neutralizing antibody assay developed by Groom et al. could not detect specific anti-XMRV antibody in 170 CFS patients [31]. These results highlight the controversy in detecting anti-XMRV antibodies due to the lack of validated assays and well-characterized reference materials.

Onlamoon et al. infected rhesus macaques with XMRV to develop an animal model of XMRV infection and to investigate the viremia and immune response against XMRV [32]. They found that viremia was detected 4 days postinfection (PI) with a peak on day 7 for one animal and a delayed and lower acute viral replication kinetic at days 14–20 in another animal [32]. The antibody responses to XMRV were observed during the second week of infection and boosted upon reexposure, but titers decreased rapidly [32, 33], indicating a weak immune response. The predominant responses were to the envelope protein gp70 and p15E, and capsid protein p30 with higher titers to gp70 and p15E than to p30, especially in acute infection of XMRV. Antibodies to gp70, p15E, and p30 persisted to 158 days and were substantially boosted by reinfection, indicating that they may be useful serologic markers for XMRV infection [33]. Based on these findings, direct chemiluminescent immunoassays (CMIAs) on the automated ARCHITECT instrument system were developed by using recombinant proteins (p15E, gp70, and p30) for both capture and detection. The prototype assays showed 100% sensitivity by detecting all Western Blot positive serial bleeds from the XMRV-infected macaques and >99% specificity with blood donors [33]. This is the first study to investigate the nature and kinetics of the antibody response to XMRV infection in animal model, and the first immunoassay that has been evaluated by the well-characterized XMRV-positive animal bleeds.

The US National Cancer Institute (NCI) has invested considerable effort in developing serological assays for detection of XMRV infection. They used the Meso Scale Discovery (MSD) platform and found that reactivity to XMRV recombinant proteins is statistically higher in XMRV-positive clinical samples from CFS patients than in normal donor plasma [34]. Conventional ELISA has been developed by using recombinant XMRV-proteins to capture antibodies in samples [30, 35]. In addition, several groups reported the use of the WB assay for detection of anti-XMRV antibodies [36, 37].

Unfortunately, all of these assays have not yet been fully validated due to lack of reference reagents for standardization of assays; therefore, it is difficult to make comparisons of results obtained in different laboratories using various-methodologies. Several groups were unable to detect XMRV-specific antibodies in PC and CFS patients as well as healthy control sera [30, 35, 36, 38]. In some studies, the weak

antibody activity could not be confirmed by other assays, such as immunofluorescence assay (IFA) or was found to be nonspecific cross-reactivity [38]. Groom et al. found that only one of the 26 samples with neutralizing activity came from a CFS patient, while 14% (22/157) blood donors were positive by their neutralizing assay [31]. However, most of the serological positive samples were also able to neutralize MLV particles pseudotyped with enveloped proteins from other viruses, including vesicular stomatitis virus, indicating significant cross-reactivity in serological responses. These results highlight the danger of overestimating XMRV frequency based on serological assays.

2.3. Other Test Methods. Both FISH and IHC were employed to localize XMRV within human prostatic tissues, and to measure the frequency of the infected cells. Urisman et al. reported that about 0.1~1.2% of the cells were positive and most FISH-positive cells were stromal fibroblasts [1]. They also used IHC to stain PC tissues with a monoclonal antibody against SFFV that was reactive against *gag* proteins from a wide range of different ecotropic, polytropic, and xenotropic MuLV strains. However, they observed that only stromal but not epithelial cells were infected with XMRV [1]. In contrast, Schlaberg et al. stained prostate tissues with anti-XMRV sera and found viral protein expression in 23% (54/233) of cases with PC and in 4% (4/101) controls. XMRV-specific staining was predominantly observed in malignant prostatic epithelial cells [23]. However, only 6 out of 54 IHC-positive PC cases were XMRV DNA positive while none of the controls was positive for the two assays. Differences between the two methods were argued to be attributed to very low viral loads, sampling differences, and varying proportions of XMRV-infected cells [23]. Aloia et al. reported no staining of 596 prostatic adenocarcinomas and 452 benign prostate tissue specimens by IHC using anti-MLV antibodies which reproducibly reacted with XMRV proteins and stained XMRV-containing cells [39]. They were unable to stain positive samples from the Schlaberg study, raising the question of whether the findings by Schlaberg et al. were valid [39]. Barnes et al. used T-cell ELISPOT assay to test responses of PBMCs to XMRV *gag* peptides as an evidence of XMRV infection of PBMCs [40]. For *ex vivo* T-cell ELISPOT assays, microtiter plates coated with anti-interferon-γ immunoglobulin G were used to incubate with XMRV peptides and PBMCs from patients, followed by incubation with biotinylated interferon-γ antibody and streptavidin-labeled chromogen. The number of reactive cells was counted. They found no positive responses to XMRV *gag* in the 63 patients infected with HIV-1 or HCV. However, patient cells were responsive to other antigens, suggesting that the absence of XMRV *gag*-specific T cells in their study. Lee described a novel cell line, Detectors of Exogenous Retroviral Sequence Elements (DERSE) indicator cells, to rapidly assess XMRV or XMLV replication in less than 3 days [14]. The DERSE cell uses a retroviral vector containing an inverted, intron-interrupted green fluorescent protein (GFP) reporter cassette. XMRV infection permitted GFP expression, which can be easily monitored by microscopy.

3. Conclusion

Currently, there are no commercially available FDA approved/licensed tests for detection of XMRV or other MLV-related human retroviruses. Standards for the diagnosis of XMRV or MLV-related retrovirus infection based on laboratory test methods have not been established. The relative sensitivity and specificity of various assay methodologies and strategies (i.e., NAT, serology, and culture) have not been determined and standards for assay performance have not yet been established. The use of multiple testing methodologies may be required because of the biology of the viruses, such as transient viremia and relatively low-immune response observed in the Macaque model. In order to avoid false-positive detection, mouse DNA contamination should be carefully examined and excluded.

Acknowledgments

The authors wish to acknowledge Dr. Krishna Devadas and Dr. Xue Wang for their review of this paper. The findings and conclusions in this paper have not been formally disseminated by the Food and Drug Administration and should not be construed to represent any agency determination of policy.

References

[1] A. Urisman, R. J. Molinaro, N. Fischer et al., "Identification of a novel Gammaretrovirus in prostate tumors of patients homozygous for R462Q RNASEL variant," *PLoS Pathogens*, vol. 2, no. 3, article e25, 2006.

[2] V. C. Lombardi, F. W. Ruscetti, J. D. Gupta et al., "Detection of an infectious retrovirus, XMRV, in blood cells of patients with chronic fatigue syndrome," *Science*, vol. 326, no. 5952, pp. 585–589, 2009.

[3] E. Callaway, "Virology: fighting for a cause," *Nature*, vol. 471, no. 7338, pp. 282–285, 2011.

[4] J. Carlowe, "Chronic fatigue syndrome is not caused by XMRV virus, study shows," *The British Medical Journal*, vol. 341, article c7358, 2010.

[5] J. M. Coffin and J. P. Stoye, "Virology. A new virus for old diseases?" *Science*, vol. 326, no. 5952, pp. 530–531, 2009.

[6] J. Cohen, "Retrovirology. More negative data for link between mouse virus and human disease," *Science*, vol. 331, no. 6022, pp. 1253–1254, 2011.

[7] M. McClure and S. Kaye, "Can detection of xenotropic murine leukemia virus-related virus be linked to chronic fatigue syndrome?" *Expert Review of Molecular Diagnostics*, vol. 10, no. 5, pp. 537–539, 2010.

[8] M. McClure and S. Wessely, "Chronic fatigue syndrome and human retrovirus XMRV," *The British Medical Journal*, vol. 340, article c1099, 2010.

[9] R. H. Silverman, C. Nguyen, C. J. Weight, and E. A. Klein, "The human retrovirus XMRV in prostate cancer and chronic fatigue syndrome," *Nature Reviews Urology*, vol. 7, no. 7, pp. 392–402, 2010.

[10] M. A. Wainberg and K.-T. Jeang, "XMRV as a human pathogen?" *Cell Host & Microbe*, vol. 9, no. 4, pp. 260–262, 2011.

[11] R. A. Weiss, "A cautionary tale of virus and disease," *BMC Biology*, vol. 8, article 124, 2010.

[12] S. C. Lo, N. Pripuzova, B. Li et al., "Detection of MLV-related virus gene sequences in blood of patients with chronic fatigue syndrome and healthy blood donors," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 36, pp. 15874–15879, 2010.

[13] R. A. Smith, "Contamination of clinical specimens with MLV-encoding nucleic acids: implications for XMRV and other candidate human retroviruses," *Retrovirology*, vol. 7, no. 1, article 112, 2010.

[14] J. P. Stoye, R. H. Silverman, C. A. Boucher, and S. F. Le Grice, "The xenotropic murine leukemia virus-related retrovirus debate continues at first international workshop," *Retrovirology*, vol. 7, no. 1, article 113, 2010.

[15] J. A. Mikovits, V. C. Lombardi, M. A. Pfost, K. S. Hagen, and F. W. Ruscetti, "Detection of an infectious retrovirus, XMRV, in blood cells of patients with chronic fatigue syndrome," *Virulence*, vol. 1, no. 5, pp. 386–390, 2010.

[16] J. A. Mikovits, Y. Huang, M. A. Pfost et al., "Distribution of xenotropic murine leukemia virus-related virus (XMRV) infection in chronic fatigue syndrome and prostate cancer," *AIDS Reviews*, vol. 12, no. 3, pp. 149–152, 2010.

[17] T. Roe, T. C. Reynolds, G. Yu, and P. O. Brown, "Integration of murine leukemia virus DNA depends on mitosis," *The EMBO Journal*, vol. 12, no. 5, pp. 2099–2108, 1993.

[18] B. P. Danielson, G. E. Ayala, and J. T. Kimata, "Detection of xenotropic murine leukemia virus-related virus in normal and tumor tissue of patients from the southern United States with prostate cancer is dependent on specific polymerase chain reaction conditions," *Journal of Infectious Diseases*, vol. 202, no. 10, pp. 1470–1477, 2010.

[19] M. J. Robinson, O. W. Erlwein, S. Kaye et al., "Mouse DNA contamination in human tissue tested for XMRV," *Retrovirology*, vol. 7, no. 1, article 108, 2010.

[20] B. Oakes, A. K. Tai, O. Cingoz et al., "Contamination of human DNA samples with mouse DNA can lead to false detection of XMRV-like sequences," *Retrovirology*, vol. 7, no. 1, article 109, 2010.

[21] E. Sato, R. A. Furuta, and T. Miyazawa, "An endogenous murine leukemia viral genome contaminant in a commercial RT-PCR Kit is amplified using standard primers for XMRV," *Retrovirology*, vol. 7, no. 1, article 110, 2010.

[22] S. Hue, E. R. Gray, A. Gall et al., "Disease-associated XMRV sequences are consistent with laboratory contamination," *Retrovirology*, vol. 7, no. 1, article 111, 2010.

[23] R. Schlaberg, D. J. Choe, K. R. Brown, H. M. Thaker, and I. R. Singh, "XMRV is present in malignant prostatic epithelium and is associated with prostate cancer, especially high-grade tumors," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 38, pp. 16351–16356, 2009.

[24] F. J. van Kuppeveld, A. S. Jong, K. H. Lanke et al., "Prevalence of xenotropic murine leukaemia virus-related virus in patients with chronic fatigue syndrome in the Netherlands: retrospective analysis of samples from an established cohort," *The British Medical Journal*, vol. 340, article c1018, 2010.

[25] K. S. Jones, Y. K. Huang, C. Petrow-Sadowski et al., "Evidence for sequeence variation in XMRV," in *Proceedings of the Annual Meeting on Retroviruses*, Cold Spring Harbor Laboratory, New York, NY, USA, abstract 13, 2010.

[26] T. Paprotka, K. A. Delviks-Frankenberry, O. Cingoz et al., "Recombinant origin of the retrovirus XMRV," *Science*, vol. 333, no. 6038, pp. 97–101, 2011.

[27] A. Dupressoir and T. Heidmann, "Expression of intracisternal A-particle retrotransposons in primary tumors of oncogene-expressing transgenic mice," *Oncogene*, vol. 14, no. 24, pp. 2951–2958, 1997.

[28] G. Simmons, S. A. Glynn, J. A. Holmberg et al., "The Blood Xenotropic Murine Leukemia Virus-Related Virus Scientific Research Working Group: mission, progress, and plans," *Transfusion*, vol. 51, no. 3, pp. 643–653, 2011.

[29] R. S. Arnold, N. V. Makarova, A. O. Osunkoya et al., "XMRV infection in patients with prostate cancer: novel serologic assay and correlation with PCR and FISH," *Urology*, vol. 75, no. 4, pp. 755–761, 2010.

[30] S. Sabunciyan, N. Mandelberg, C. S. Rabkin, R. Yolken, and R. Viscidi, "No difference in antibody titers against xenotropic MLV related virus in prostate cancer cases and cancer-free controls," *Molecular and Cellular Probes*, vol. 25, no. 2-3, pp. 134–136, 2011.

[31] H. C. T. Groom, V. C. Boucherit, K. Makinson et al., "Absence of xenotropic murine leukaemia virus-related virus in UK patients with chronic fatigue syndrome," *Retrovirology*, vol. 7, no. 1, article 10, 2010.

[32] N. Onlamoon, J. Das Gupta, P. Sharma et al., "Infection, viral dissemination and antibody responses of rhesus macaques exposed to the human gammaretrovirus XMRV," *The Journal of Virology*, vol. 85, no. 9, pp. 4547–4557, 2011.

[33] X. Qiu, P. Swanson, K. C. Luk et al., "Characterization of antibodies elicited by XMRV infection and development of immunoassays useful for epidemiologic studies," *Retrovirology*, vol. 7, no. 1, article 68, 2010.

[34] R. K. Bagni, K. Beam, A. Meade et al., "Development and optimization of a multiplex serological assay to detect XMRV antibodies," in *Proceedings of the 1st International Workshop on XMRV*, abstract O_14, 2010.

[35] O. Hohn, H. Krause, P. Barbarotto et al., "Lack of evidence for xenotropic murine leukemia virus-related virus(XMRV) in German prostate cancer patients," *Retrovirology*, vol. 6, no. 1, article 92, 2009.

[36] W. M. Switzer, H. Jia, O. Hohn et al., "Absence of evidence of xenotropic murine leukemia virus-related virus infection in persons with chronic fatigue syndrome and healthy controls in the United States," *Retrovirology*, vol. 7, no. 1, article 57, 2010.

[37] B. C. Satterfield, R. A. Garcia, H. Jia et al., "Serological and PCR testing of persons with chronic fatigue syndrome in the United States shows no association with xenotropic or polytropic murine leukemia virus-related viruses," *Retrovirology*, vol. 8, no. 1, article 12, 2011.

[38] O. Hohn, K. Strohschein, A. U. Brandt et al., "No evidence for XMRV in German CFS and MS patients with fatigue despite the ability of the virus to infect human blood cells in vitro," *PLoS One*, vol. 5, no. 12, article e15632, 2010.

[39] A. L. Aloia, K. S. Sfanos, W. B. Isaacs et al., "XMRV: a new virus in prostate cancer?" *Cancer Research*, vol. 70, no. 24, pp. 10028–10033, 2010.

[40] E. Barnes, P. Flanagan, A. Brown et al., "Failure to detect xenotropic murine leukemia virus-related virus in blood of individuals at high risk of blood-borne viral infections," *Journal of Infectious Diseases*, vol. 202, no. 10, pp. 1482–1485, 2010.

Hindawi Publishing Corporation
Advances in Virology
Volume 2011, Article ID 940210, 10 pages
doi:10.1155/2011/940210

Review Article

Endogenous Murine Leukemia Viruses: Relationship to XMRV and Related Sequences Detected in Human DNA Samples

Oya Cingöz and John M. Coffin

Department of Molecular Biology and Microbiology and Genetics Program, Sackler School of Graduate Biomedical Sciences, Tufts University, Boston, MA 02111, USA

Correspondence should be addressed to John M. Coffin, john.coffin@tufts.edu

Received 15 June 2011; Accepted 7 July 2011

Academic Editor: Arifa S. Khan

Xenotropic-murine-leukemia-virus-related virus (XMRV) was the first gammaretrovirus to be reported in humans. The sequence similarity between XMRV and murine leukemia viruses (MLVs) was consistent with an origin of XMRV from one or more MLVs present as endogenous proviruses in mouse genomes. Here, we review the relationship of the human and mouse virus isolates and discuss the potential complications associated with the detection of MLV-like sequences from clinical samples.

1. Endogenous MLVs

Retroviruses are unique in their requirement, as a natural step in their replication cycle, for integration into the genomes of the host cells they infect [1]. Through infection of the germ line, exogenous retroviruses can become a part of the host genome, leading to the generation of endogenous proviruses. All vertebrate species examined carry remnants of such prior retroviral infections in their genomes [1, 2]. Humans, for example, carry some 80,000 sequences or about 8% of our total genome, derived from retrovirus infections dating from some 40 million to a hundred thousand or so years ago. Mouse genomes contain a large number of more recently integrated endogenous proviruses; one of the best-studied groups comprises the murine leukemia viruses (MLVs). MLVs are thought to have entered the *Mus* germ line less than 1.5 million years ago [3, 4], and generation of new endogenous proviruses continues to this day. They can be classified based on their host range and sequence into ecotropic, xenotropic, polytropic and modified polytropic, MLVs [1, 5]. The host range of MLVs is determined by the species distribution of the receptors they use for entry. Ecotropic MLVs (of which there are only a few) use mCAT1, an allele of the cationic amino acid transporter found only in mice and a few other rodents [6]. The more common nonecotropic MLVs, including xenotropic (X-MLV), polytropic (P-MLV), and modified polytropic (MP-MLV) viruses use distinct alleles of Xpr1, a cell surface protein of unknown function, with X-MLVs unable to recognize the allele found in most inbred and a few wild mice, hence the name "xenotropic" (reviewed in [7]). The proviruses that correspond to these different viruses are referred to as *Xmv*, *Pmv*, and *Mpmv*, respectively. Experimentally, they are distinguished by hybridization to oligonucleotide probes specific for a sequence in the SU region of *env*. P-MLVs can be distinguished from MP-MLVs by a 27 bp insertion in their *env* genes [1, 5]. Endogenous nonecotropic proviruses are highly polymorphic in their genome location: inbred mice contain about 20 of each type and, on average, share about 30 with any other inbred strain. Of the 150 or so identified proviruses, only a few *Xmvs*, including *Bxv1* (*Xmv43*), are known to encode infectious virus [8]. No complete, replicating P-MLV or MP-MLV has ever been identified, although their *env* genes are often found as recombinants [1, 9].

As is the case with most host-pathogen arms races, evolution at the host-virus interface for MLVs and their mouse hosts is apparent on multiple levels. *Xmv* proviruses are considered to be the oldest MLV subgroup, a hypothesis supported by the genetic diversity among members, their failure to form a monophyletic clade, and their ancestral location in phylogenetic analyses (Figure 1) [10]. During the long course of their coexistence with endogenous and exogenous

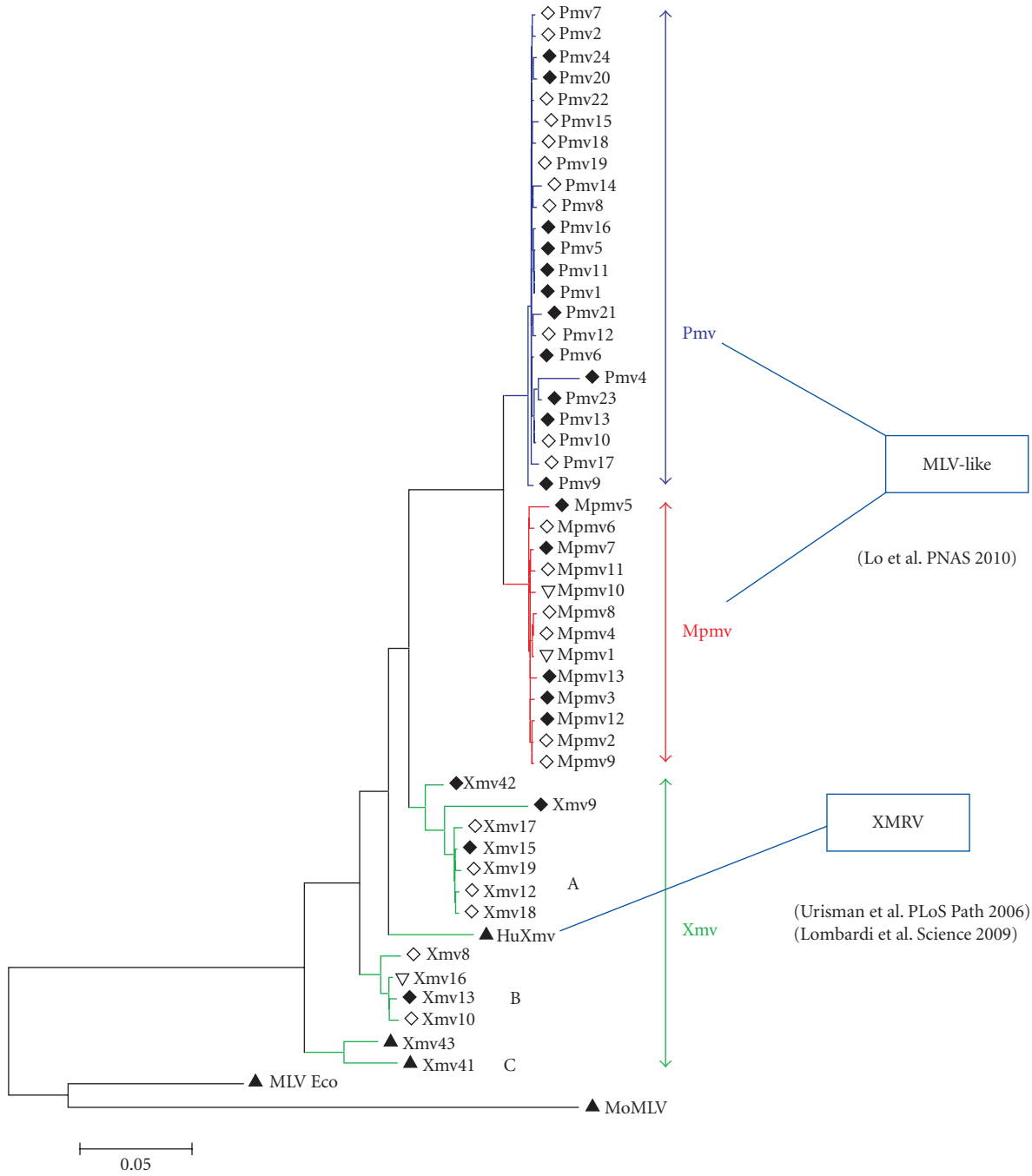


Figure 1: Relationship of proviruses in the sequenced mouse genome. The three groups of nonecotropic MLVs are indicated. Modified from Jern et al. [10].

MLVs, many *Mus* subspecies have evolved ways to cope with such assaults. One example of a resistance mechanism is the evolution of variants of the Xpr1 receptor so that the modified allele no longer supports virus entry, conferring a selective advantage on the host that carries the new variant. Endogenous X-MLVs derived from proviruses in those mice could not infect the cells of their host organism. Viruses have in turn responded by incorporating mutations in their *env* genes, allowing them to use the new version of the receptor and giving rise to the evolution of polytropic and modified polytropic MLVs, which are also carried in numerous copies by many mouse genomes (Figure 2). Another level of resistance is encoded by a set of proteins induced as result of the expression of interferon (reviewed in [11]). One of the most important of these is the enzyme family APOBEC3 A-G (in humans) with one homolog in mice, Apobec3. APOBEC3's are incorporated into virions and cause high levels of dC to dU deamination on newly synthesized negative

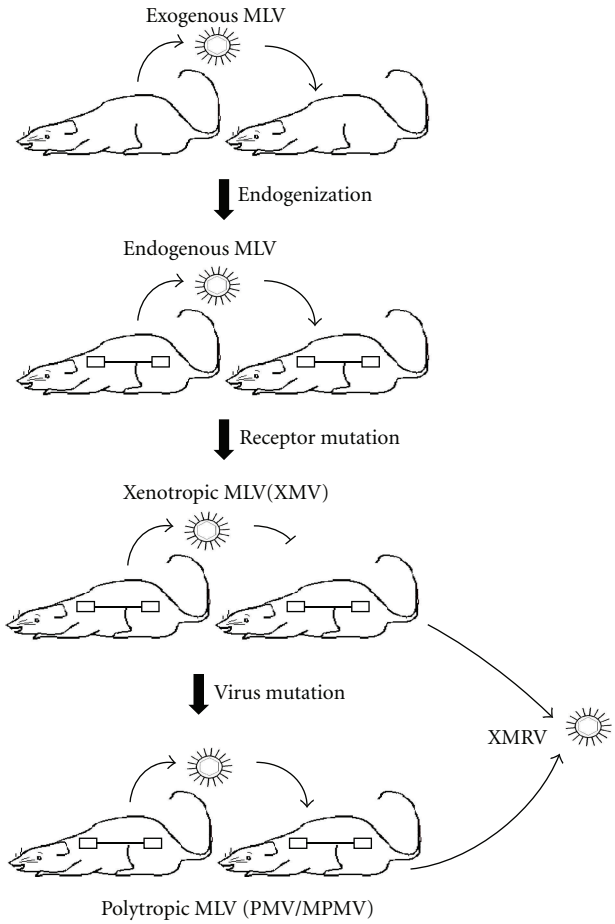


Figure 2: Schematic cartoon of the hypothetical origin of X-MLV, P-MLV, and XMRV. The mice illustrate the inferred pattern of MLV-host evolution, starting with infection of mice with an exogenous XMLV, its endogenization, selection for the resistant ($Xpr1^n$) receptor allele, and the subsequent evolution of the polytropic (P-MLV and MP-MLV) viruses capable of using the mutant receptor.

strand DNA, leading to substantial frequencies of G to A hypermutation in the positive strand (genome) of many retroviruses, effectively killing the provirus.

2. Origin of XMRV

Evidence for the presence of XMRV and other MLV-related viruses in human tissues have so far relied on various methods including fluorescence in situ hybridization (FISH), immunological detection of viral antigens and antibodies against them, isolation of infectious virus from human samples, and, finally, PCR amplification of MLV sequences [12–14]. We will discuss here only the last two issues, focusing on the relationship of XMRV and related sequences to endogenous MLVs and the likely events that gave rise to them. By the term "XMRV," we refer only to the infectious viruses reported in the original papers [12, 13, 15]; related sequences detected by PCR amplification [14] will be referred to as "MLV-like" (Figure 1). We first focus on XMRV.

XMRV was originally described in a fraction of prostate cancer cases [12] and subsequently in a large fraction of patients with chronic fatigue syndrome (CFS) [13]. The association of XMRV with disease rapidly became controversial, however, when a large number of the studies in multiple patient cohorts, prompted by the initial reports, did not find XMRV, even though very sensitive detection methods were used (reviewed in [16]). The ensuing debate raised the issue of whether, on the one hand, the negative studies reflected poor technique or inappropriate study cohorts, or on the other, the positive results reflected contamination with a virus that did not, in fact, circulate in humans.

In contrast to most other irreproducible reports of human "rumor" viruses dating back to the 1970s, XMRV was isolated as an infectious virus and grows to high titer on suitable human cell lines, from both diseases [13, 17]. An essentially identical virus was also found to be produced in large amounts by the 22R*v1* cell line [15], which had been derived from a human prostate cancer by repeated passage, over the course of 7 years, in the form of xenografts, in nude mice (Figure 3) [18]. This result was interpreted to imply that the tumor that eventually gave rise to the cell line was infected with XMRV at the outset.

XMRV shows high sequence similarity to endogenous *Xmv* proviruses [12], suggestive of a shared origin, but it is not identical to any of them. Despite considerable effort, we did not find it as a single endogenous provirus in any mouse genome [19]. However, the fortunate availability of DNA samples from various passages of the tumor xenografts from which 22R*v1* had been derived made it possible to trace the origin of XMRV [19]. Analysis of DNAs from early and late passages revealed that the virus was undetectable (less than about 1 provirus per 200 cells) in samples taken through 1993, showing that the original tumor did not contain XMRV (Figure 3). However, it was present in the xenografts performed after 1996, suggesting that the tumor had been infected by XMRV while being passaged through nude mice some time between 1993 and 1996. Moreover, two XMRV-related proviruses (PreXMRV-1 and -2) could be detected in the small amount of mouse DNA in the tumor samples, showing that the mouse strains used for the xenografts contained these two previously undescribed proviruses, whose genomes could recombine to generate a virus virtually identical to XMRV (Figure 4), providing the most likely explanation of how, where, and when the virus was created [19]. Retrovirus recombination is a very frequent event that occurs following infection of a cell with a virion containing two distinct genomes, produced by a cell containing the two parental proviruses. During the course of reverse transcription, the enzyme transfers repeatedly from one genome to the other, on average 4 times in the case of MLVs [20]. In the case of XMRV, a virion containing the PreXMRV-1 and PreXMRV-2 genomes and most likely containing proteins made by both proviruses and produced by a cell of the mouse host would have infected a human cell in the xenograft, leading to the infectious XMRV recombinant. The virus generated in this way could then spread through the tumor, perhaps conferring some selective advantage (such as increased growth rate or hormone independence) to the infected cells. Given the large number of stretches of identical sequence in the two parental proviruses, the probability of

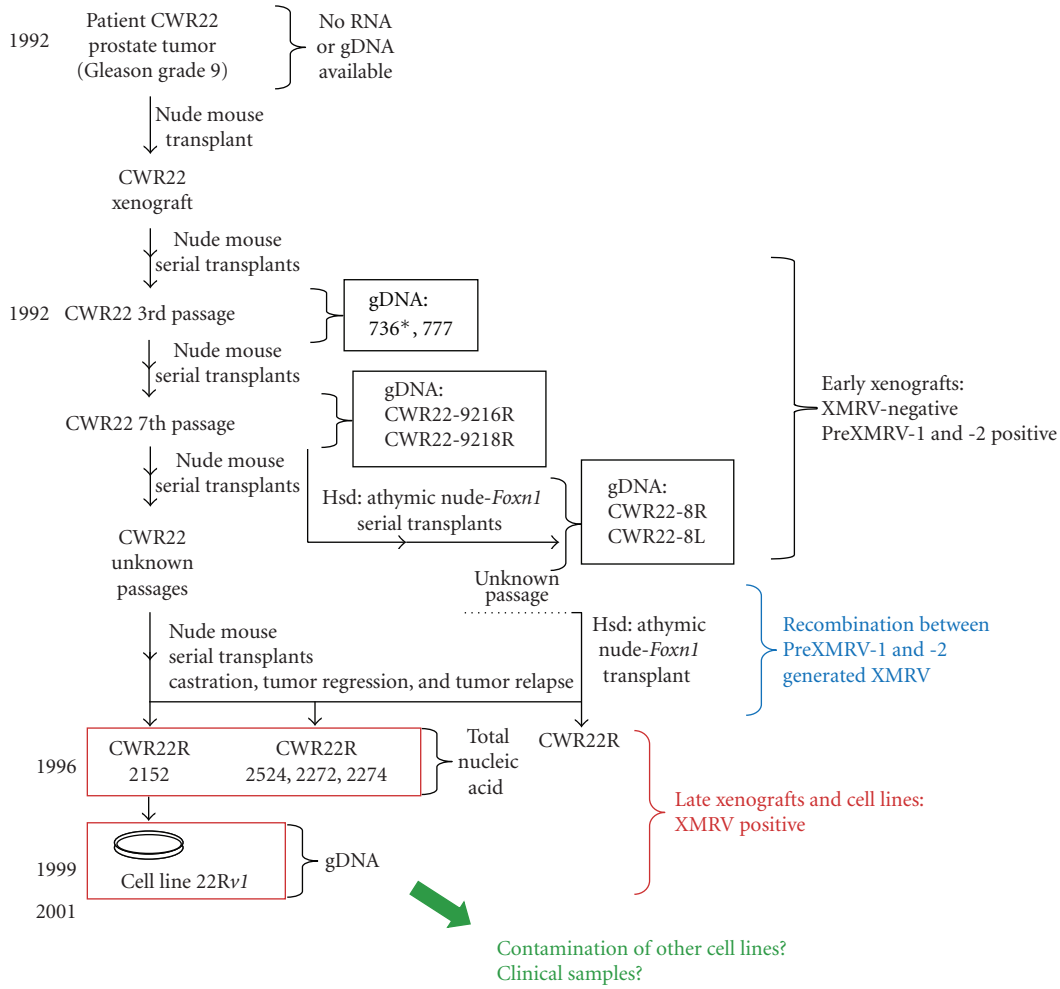


Figure 3: Derivation of the 22R*v1* cell line from prostate cancer xenografts in nude mice. Starting in 1992, the CWR22 prostate cancer was passaged repeatedly in nude mice until 1999, when the cell line was isolated. Samples of the tumor prior to 1996 were negative for XMRV, but contained small amounts of mouse DNA harboring both PreXMRV-1 and -2. Later samples were also positive for XMRV. Modified from Paprotka et al. [19].

generating exactly the same recombinant more than once independently is negligible. These findings lead inexorably to the conclusion that the XMRV produced by 22R*v1* cells was created in the laboratory (or, more likely, the mouse room) and all subsequent isolates are likely to have originated from this single unique recombination event, almost certainly by cross-contamination in laboratories handling 22R*v1* cells and other susceptible cell lines.

As mentioned above, xenotropic MLVs typically cannot use the nonpermissive Xpr1^n receptor variant carried by most inbred strains [7]. When they encounter a permissive cell type, however, as happens when human cells are transplanted into mice, they can infect the cells of this newly encountered species. Acquisition of mouse endogenous retroviruses by heterologous cells occurs frequently, and many examples have been documented [9, 21–24]. Thus, the infection of the prostate cancer xenograft by an X-MLV in mice was far from unprecedented, but the details of the process, including the specific proviruses involved, had never before been worked out.

In the decade following its derivation in 1999 [18], the 22R*v1* cell line has been distributed worldwide and widely used for studies on prostate cancer biology. At the time of this publication, PubMed listed over 200 papers reporting its use from many different laboratories, none of which could have been aware before 2009 that it was producing 10^9–10^{10} of virions per mL [15]. Given the ease with which viruses can spread from one culture to another even in virology laboratories that are aware of the problem, it is not hard to see how XMRV could have contaminated cultures in many different laboratories. Such contamination not only can give rise to false associations with human disease, but also, cause major changes in cellular processes, leading to alterations in cellular pathways under study. For these reasons, it is absolutely necessary, and should be routine practice, to continuously monitor cell lines for contaminating retroviruses.

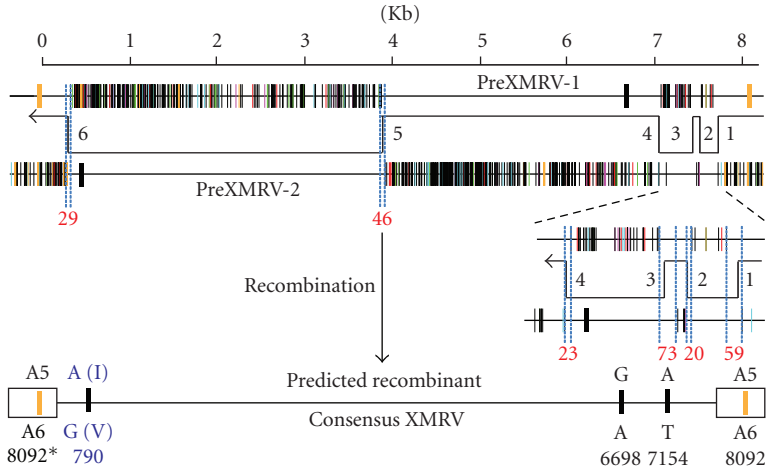


Figure 4: Recombination between PreXMRV-1 and -2 leads to XMRV. The sequences of the two proviruses identified by Paprotka et al. are shown schematically, with a vertical line indicating each position that differs from the XMRV consensus. The red line shows the positions of the 6 crossover events (common during retrovirus replication) that are inferred to have given rise to a virus differing from XMRV at only the 4 positions shown. Modified from Paprotka et al. [19].

Phylogenetic analysis of XMRV isolates in comparison with endogenous MLVs also strongly supports the same series of events. As can be seen in Figure 5, PreXMRV-1 groups with another *Xmv* subclade; PreXMRV-2 (which may itself be a recombinant) is closer, but not identical, to *Pmvs*. In the enlarged portion of the tree representing all published XMRV isolates, it can be seen that the inferred recombinant virus occupies a position ancestral to all XMRVs, followed by the virus produced by 22R*v1* cells, followed by the prostate cancer isolates, and followed finally by the CFS isolates, exactly consistent with the inferred sequence of infection and contamination events [19].

The two XMRV parents are not unique to nude mice: of 48 laboratory mouse strains tested, PreXMRV-1 is found in 6, PreXMRV-2 in 25 [Cingöz et al. In prep.], as well as in the NIH3T3 cell line, commonly used for many laboratory purposes, including preparation of MLV-based gene therapy vectors [25]. Three laboratory strains, including the two nude mice presumably used to passage the original tumor, contain both proviruses. They are also found in DNA from some wild-derived mice: PreXMRV-1 in *M. m. molossinus* and *M. m. castaneus*, and PreXMRV-2 in *M. m. domesticus*. Since the former mice are native to Asia, and the latter to Europe, it is improbable that they were ever together in the wild before human intervention.

In further support of the idea that XMRV has not infected humans is its high sensitivity to the primate APOBEC3G restriction factor, an interferon-inducible protein, which is constitutively expressed in some (but not all) cell types. Indeed, the extensive hypermutation caused by A3G makes it impossible to establish spreading infection of human PBMCs in cell culture [26] and, while proviral DNA persists in macaques experimentally infected with XMRV, this DNA is also heavily hypermutated [Kearney et al. In prep.]. Unlike PBMCs, a number of human cell lines, including the prostate cancer lines 22R*v1* and LNCaP do not express APOBEC3G. Combined with a favorable transcriptional environment [17], this property makes these cells particularly good hosts for XMRV infection, so it is not impossible that the virus could sometimes bypass the barrier to infect of blood cells.

3. MLV-Like Sequences

In an attempt to replicate the findings of XMRV in CFS patients, Lo et al. analyzed plasma and PBMCs from another cohort of CFS patients as compared to samples obtained at a different time and place from normal blood donors [14]. They reported that they were unable to detect XMRV using a specific PCR assay, but that, with somewhat less selective primers, they could detect fragments of sequence identical or closely related to some endogenous P-MLVs and MP-MLVs. Again, the sequences were detected much more frequently in samples from CFS patients than from the poorly matched controls. The close match of these sequences to endogenous MLVs present in over 100 copies per cell in laboratory and wild mice immediately raised the possibility of contamination of the samples with traces of mouse DNA. To counter this concern, Lo et al. also tested the samples for mouse mitochondrial DNA and, finding none, concluded that the results reflected infection of the patients, but not the controls, with polytropic-like MLV. In further support of this conclusion, they reported, that later samples from some of the same patients also contained MLV-like sequences. These sequences showed genetic differences from the earlier ones, leading them to conclude that there was ongoing virus replication and evolution.

The possibility of a human gammaretrovirus circulating among the population and potentially having an association with human disease created considerable excitement in the field. A number of researchers proposed that XMRV- and MLV-related sequences represented related findings that

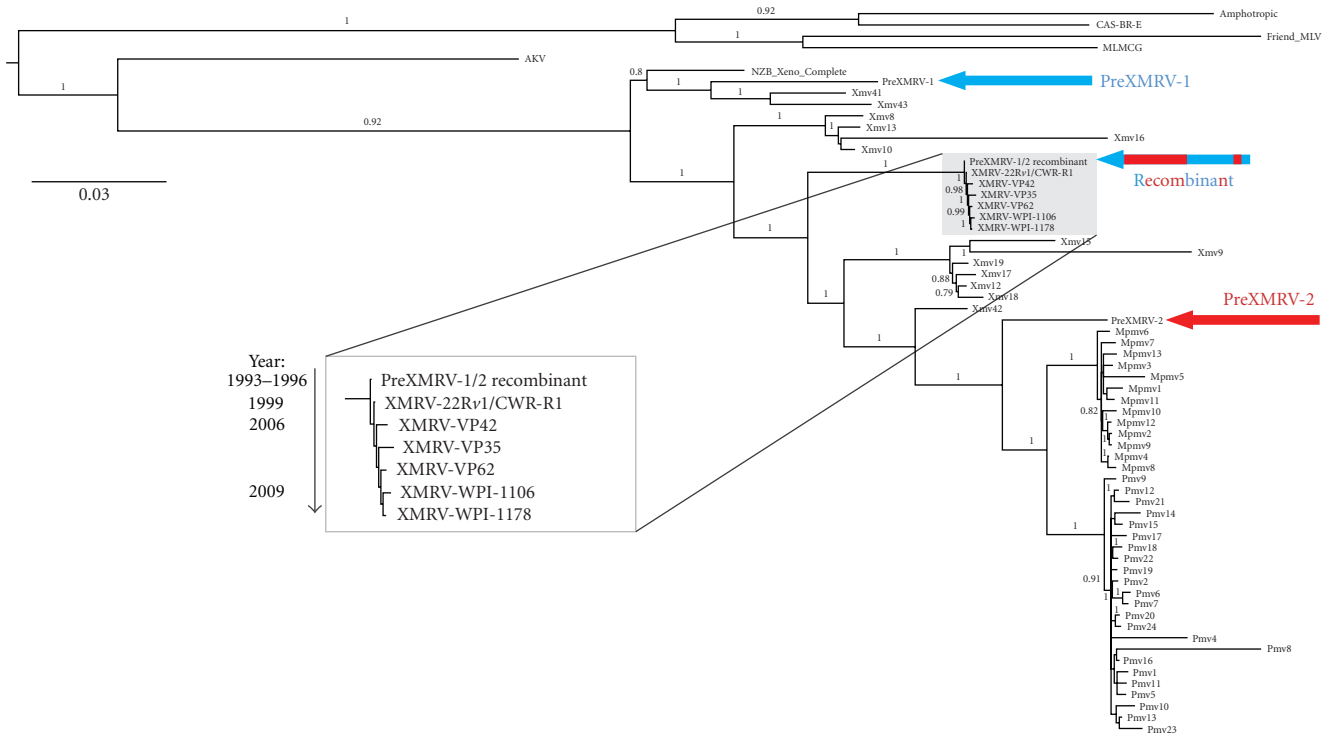


Figure 5: Phylogenetic analysis of MLVs and XMRV isolates. Note the positions of the two parents. The enlarged inset shows the XMRV portion of the tree, illustrating the ancestral position of the inferred recombinant and the subsequent possible progression of the virus. From Paprotka et al. [19].

strongly supported the conclusion of the association of MLV-like viruses with human disease. However, one should be very cautious about associating the two observations. Xenotropic and polytropic viruses are distinct MLV subclasses, a fact readily observed upon comparing the positions they occupy in phylogenetic trees [10] (Figures 1 and 5). XMRV is not found as a single endogenous provirus in any mouse strain tested [19], whereas the partial sequences detected by Lo et al. are very close or identical to proviruses found in mice [14]. XMRV sequences detected by Lombardi et al. are nearly identical to the XMRV sequences described by Urisman et al. and form a distinct clade among *Xmv* proviruses, while the MLV-like sequences of Lo et al. found in CFS and control samples are dispersed among other mouse endogenous proviruses [12–14] (Figure 6). The reported MLV-like sequences are bulk PCR fragments; no full length viral genome or infectious virus was recovered, as opposed to the Lombardi et al. study where infectious virus was recovered upon culturing patient samples. P-MLV fragments were detected following high numbers of PCR amplification cycles; no other detection methods were used and the results have not been replicated by any other study published to date. The differences between the two studies in the experimental methods used and the findings reported call for extreme caution to be taken before widely interpreting the data. Until we have a better understanding about the relationship between these viruses, the two studies should be treated separately and should not be taken as supporting or refuting each other.

Despite the inclusion of apparently adequate controls, mouse DNA contamination remains a significant concern. Extremely small amounts of DNA, from as little as one one-hundredth of a cell, contain enough provirus sequences to yield false positives with internal provirus-specific primers (Figure 6, middle panel). Two recent studies have described the detection of MLV-like sequences in samples from patients and/or healthy controls. In the first study by Oakes et al., positive amplification results were obtained from only 1 out of 111 samples tested from CFS patients, but from 18/36 healthy controls, which had been processed at a different time with a slightly different protocol [27]. In the second study, Robinson et al. found that 14/282 of UK prostate cancer cases, 5/139 of Korean, and 2/6 of Thai cases tested positive for amplification with XMRV primers [28]. As with the Lo et al. sequences, those of Oakes and Robinson fit perfectly within the endogenous MLV phylogeny (Figure 6, left panel). In both cases, some, but not all, MLV-positive samples were also positive for mouse mitochondrial DNA. To improve the sensitivity of detection of mouse DNA, we developed an assay that relies on PCR amplification of a small fragment from the LTR of intracisternal A particles (IAPs), LTR retroelements that are abundant (more than 1000 copies) in the mouse genome and not cross-reactive with any sequence in the human genome despite the presence of distantly related IAP elements [29]. Using this assay, both studies found that all samples that tested positive for MLV DNA sequences were also positive for IAP LTR sequences, implying sporadic mouse DNA contamination as the most likely source of the

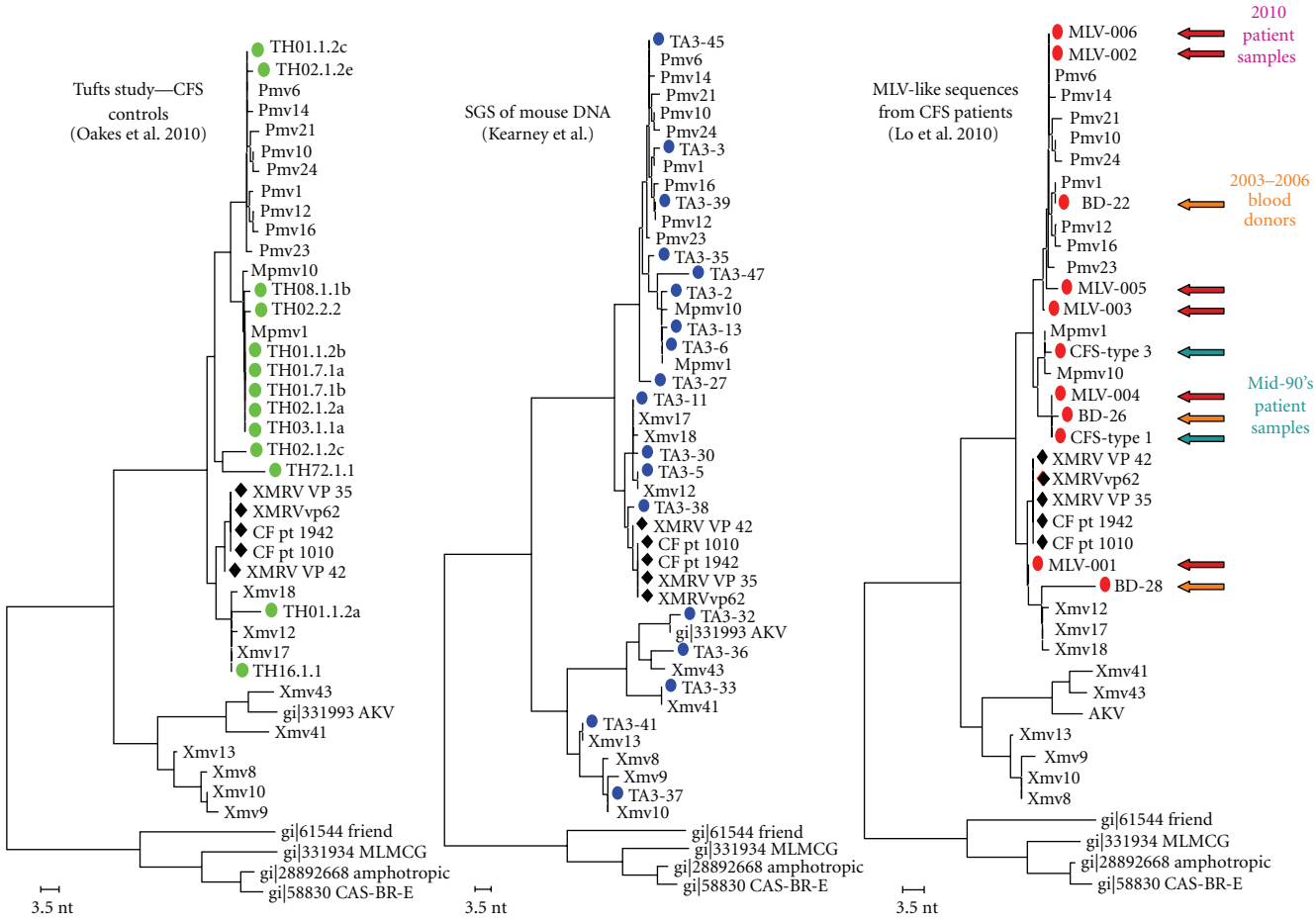


Figure 6: Phylogenetic analysis of MLV-like sequences. The three trees relate the endogenous MLV sequences described by Jern et al. [10] to the fragmentary MLV-like sequences found in (left, green dots) normal control DNAs by Oakes et al. [27], in (middle, blue dots) normal mouse DNA by M. Kearney (unpublished), and (right, red dots) in CFS samples (green arrows), normal blood donors (orange arrows), and samples drawn at a later date from the same CFS patients (red arrows) (data from Lo et al. [14]).

former sequences. The exact source of these mouse sequences has not fully pinned down. Trace amounts of mouse DNA could be already present in the laboratory reagents used or contamination could have occurred during sample collection or storage before they were even sent to the laboratories to be tested for XMRV. Four other studies further supported these findings, where potential sources of MLV genome contaminants, most likely from mouse genomic DNA, were discovered in commercially available laboratory reagents and kits, particularly Taq DNA polymerase containing a mouse monoclonal antibody [30–33]. It is possible that microtomes used for both laboratory and clinical samples could carry traces of mouse DNA over to human pathology samples, perhaps including the fixed and archived prostate cancer specimens analyzed by Robinson et al. [28]. Cross-contamination from a laboratory robot used for XMRV more than a year previously has also been reported [33]. Taken together, these findings call for caution before interpreting the data and the need for very sensitive assays to detect mouse DNA contamination, when endogenous MLV sequences are detected by PCR from human samples.

It is also important to emphasize that considerable caution should be exercised when attributing the origin of short PCR amplicons to a replicating virus, when the virus has itself not been isolated. Indeed, for reasons that are unclear, replication-competent P-MLVs have never been identified in mice, although their envelope genes can be donated to replication-competent recombinant viruses that arise and cause lymphoma in some strains of mice [35–37]. Until a real virus is identified, studies reporting detection of "virus" sequences must be taken as highly preliminary and suggestive, not definitive, evidence for human infection by P-MLVs.

A final problem with the sequences reported by Lo et al. is that they do not exhibit the sort of evolution expected for long-term infection. Although these authors reported that sequences that appeared to show evolutionary changes were obtained from some of the same patients ~15 years after the initial sampling [14], examination of the sequences made available in the GenBank database reveals that the "evolved" sequences are in fact very similar to other endogenous MLVs. (Figure 6, right panel) [27]. Rather than evolving as would be expected of a true infecting virus, these sequences

therefore seem to be simply moving up and down the MLV phylogenetic tree. The conclusion that they represent MLV sequences amplified from trace mouse DNA contamination is inescapable.

4. Particular Issues regarding Detection of MLVs as Possible Human Pathogens

The initial reports of association between endogenous MLV-related viruses and human disease were attractive because of their biological plausibility: MLV-related viruses cause a similar variety of diseases in mouse models [1]; close contact between mice and humans can result in zoonotic infection; the virus isolated is highly infectious for at least some human tumor cell lines [17, 38]. As the studies presented here unfolded, however, a number of experimental issues regarding the possibility of detection of endogenous proviral sequences and their confusion with replicating viruses infecting human patients came to light. There are a number of lessons that should be heeded in the development of future studies.

First, low levels of mouse DNA contamination are very commonly found in laboratory reagents. In some cases, this DNA can be attributed to the inclusion of mouse-derived products, such as monoclonal antibodies in PCR reagents, or used for isolation of cells [27, 28, 30, 32, 33]; in others, the source of mouse DNA is less than clear, but given the close relationship of human and wild mouse activity, it is not hard to imagine that mice can leave traces in many places that can find their way into almost any laboratory reagent or supply.

A second, related issue regards the provision of appropriate controls. Given the apparent sporadic nature of this sort of contamination, it is absolutely essential that controls and patient samples be exactly matched, not only for personal characteristics, such as age, gender, geography, and so forth, but also in the reagents and materials (tubes, needles, etc.) used to obtain and process the assay samples. Unfortunately, such caution is often not the case, particularly in retrospective studies [14]. Clinical samples are often collected as at least two separate groups, the simplest example being patient versus control samples. This lack of caution can result in detection of a contaminant in one set of samples and not the other, resulting in false association of virus with human disease. Differential association between two different groups of clinical specimens can occur, even if the detected entity is an artifact. In fact, in the study by Oakes et al. [27], MLV sequences were detected preferentially in healthy controls drawn at a later time and processed by a slightly different method. One possible explanation for such erroneous associations is that the two groups might have been handled differently, collected at different times by different people at different locations using different reagents, supplies, or methods. Furthermore, tubes containing patient samples may have been accessed more frequently and under different circumstances than controls. For these reasons, blinding of investigators to which samples are cases and which are controls for such studies is crucial. Examples of such false associations have persisted, even when independent laboratories had confirmed findings after the initial report [39].

Third, even in the absence of contaminating mouse DNA, a different complication arises from retrovirus-contaminated cell lines used in the laboratory. There are multiple documented cases of such contamination events, which appear to be quite common among laboratories working with retroviruses [8, 15, 40–43]. Even in laboratories that do not work with retroviruses, there are examples of cell lines producing replication-competent retroviruses. Contamination of cell lines with retroviruses could occur through cross-contamination of previously uninfected cells with a virus grown or handled on other cells nearby. As long as the virus is replication-competent and can establish an efficient infection, it could eventually take over the entire culture even with trace amounts of starting virus.

The overall lesson to be learned here is that extreme measures are required to avoid false associations of mouse viruses with disease, including: (1) rigorous use of highly sensitive assays for detecting mouse DNA contamination of supplies and reagents; (2) frequent testing of cell cultures used in the laboratory for undetected infection with an MLV or another virus; (3) the use of controls that are exactly contemporaneous to the cases and obtained by precisely the same methods using the same materials and reagents. As a few recent papers indicate [30, 32, 33], these conditions are not easy to achieve, but only laboratories that do so can make credible claims to the discovery of new human infections.

Acknowledgments

The authors thank Mary Kearney for help with the analyses shown in Figure 6. This work was supported by research Grant R37 CA 089441 to J. M. Coffin. J. M. Coffin was a Research Professor of the American Cancer Society, with support from the F. M. Kirby Foundation.

References

[1] J. M. Coffin, S. H. Hughes, and H. Varmus, *Retroviruses*, Cold Spring Harbor Laboratory Press, Plainview, NY, USA, 1997.

[2] E. Herniou, J. Martin, K. Miller, J. Cook, M. Wilkinson, and M. Tristem, "Retroviral diversity and distribution in vertebrates," *Journal of Virology*, vol. 72, no. 7, pp. 5955–5966, 1998.

[3] K. Tomonaga and J. M. Coffin, "Structures of endogenous nonecotropic murine leukemia virus (MLV) long terminal repeats in wild mice: implication for evolution of MLVs," *Journal of Virology*, vol. 73, no. 5, pp. 4327–4340, 1999.

[4] C. Stocking and C. A. Kozak, "Endogenous retroviruses: murine endogenous retroviruses," *Cellular and Molecular Life Sciences*, vol. 65, no. 21, pp. 3383–3398, 2008.

[5] J. P. Stoye and J. M. Coffin, "The four classes of endogenous murine leukemia virus: structural relationships and potential for recombination," *Journal of Virology*, vol. 61, no. 9, pp. 2659–2669, 1987.

[6] L. M. Albritton, L. Tseng, D. Scadden, and J. M. Cunningham, "A putative murine ecotropic retrovirus receptor gene encodes a multiple membrane-spanning protein and confers susceptibility to virus infection," *Cell*, vol. 57, no. 4, pp. 659–666, 1989.

[7] C. A. Kozak, "The mouse "xenotropic" gammaretroviruses and their XPR1 receptor," *Retrovirology*, vol. 7, no. 1, article 101, 2010.

[8] K. S. Sfanos, A. L. Aloia, J. L. Hicks et al., "Identification of replication competent murine gammaretroviruses in commonly used prostate cancer cell lines," *PLoS ONE*, vol. 6, no. 6, article e20874, 2011.

[9] J. W. Hartley, N. K. Wolford, L. J. Old, and W. P. Rowe, "A new class of murine leukemia virus associated with development of spontaneous lymphomas," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 74, no. 2, pp. 789–792, 1977.

[10] P. Jern, J. P. Stoye, and J. M. Coffin, "Role of APOBEC3 in genetic diversity among endogenous murine leukemia viruses," *PLoS Genetics*, vol. 3, no. 10, article e183, pp. 2014–2022, 2007.

[11] S. Neil and P. Bieniasz, "Human immunodeficiency virus, restriction factors, and interferon," *Journal of Interferon and Cytokine Research*, vol. 29, no. 9, pp. 569–580, 2009.

[12] A. Urisman, R. J. Molinaro, N. Fischer et al., "Identification of a novel gammaretrovirus in prostate tumors of patients homozygous for R462Q RNASEL variant," *PLoS Pathogens*, vol. 2, no. 3, article e25, 2006.

[13] V. C. Lombardi, F. W. Ruscetti, J. D. Gupta et al., "Detection of an infectious retrovirus, XMRV, in blood cells of patients with chronic fatigue syndrome," *Science*, vol. 326, no. 5952, pp. 585–589, 2009.

[14] S. C. Lo, N. Pripuzova, B. Li et al., "Detection of MLV-related virus gene sequences in blood of patients with chronic fatigue syndrome and healthy blood donors," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 36, pp. 15874–15879, 2010.

[15] E. C. Knouf, M. J. Metzger, P. S. Mitchell et al., "Multiple integrated copies and high-level production of the human retrovirus XMRV (Xenotropic Murine leukemia virus-Related Virus) from 22Rv1 prostate carcinoma cells," *Journal of Virology*, vol. 83, no. 14, pp. 7353–7356, 2009.

[16] M. Cornelissen, F. Zorgdrager, P. Blom et al., "Lack of detection of XMRV in seminal plasma from HIV-1 infected men in The Netherlands," *PLoS ONE*, vol. 5, no. 8, article e12040, 2010.

[17] J. J. Rodriguez and S. P. Goff, "Xenotropic murine leukemia virus-related virus establishes an efficient spreading infection and exhibits enhanced transcriptional activity in prostate carcinoma cells," *Journal of Virology*, vol. 84, no. 5, pp. 2556–2562, 2010.

[18] R. M. Sramkoski, T. G. Pretlow, J. M. Giaconia et al., "A new human prostate carcinoma cell line, 22Rv1," *In Vitro Cellular and Developmental Biology—Animal*, vol. 35, no. 7, pp. 403–409, 1999.

[19] T. Paprotka, K. A. Delviks-Frankenberry, O. Cingöz et al., "Recombinant origin of the retrovirus XMRV," *Science*, vol. 333, no. 6038, pp. 97–101, 2011.

[20] J. Zhuang, S. Mukherjee, Y. Ron, and J. P. Dougherty, "High rate of genetic recombination in murine leukemia virus: implications for influencing proviral ploidy," *Journal of Virology*, vol. 80, no. 13, pp. 6706–6711, 2006.

[21] T. S. Tralka, C. L. Yee, A. B. Rabson et al., "Murine type C retroviruses and intracisternal A-particles in human tumors serially passaged in nude mice," *Journal of the National Cancer Institute*, vol. 71, no. 3, pp. 591–599, 1983.

[22] J. A. Levy and T. Pincus, "Demonstration of biological activity of a murine leukemia virus of New Zealand black mice," *Science*, vol. 170, no. 3955, pp. 326–327, 1970.

[23] B. G. Achong, P. A. Trumper, and B. C. Giovanella, "C type virus particles in human tumours transplanted into nude mice," *British Journal of Cancer*, vol. 34, no. 2, pp. 203–206, 1976.

[24] H. Wunderli, D. D. Mickey, and D. F. Paulson, "C-type virus particles in human urogenital tumours after heterotransplantation into nude mice," *British Journal of Cancer*, vol. 39, no. 1, pp. 35–42, 1979.

[25] R. Mendoza, A. E. Vaughan, and A. D. Miller, "The left half of the XMRV retrovirus is present in an endogenous retrovirus of NIH/3T3 swiss mouse cells," *Journal of Virology*, vol. 85, no. 17, pp. 9247–9248, 2011.

[26] T. Paprotka, N. J. Venkatachari, C. Chaipan et al., "Inhibition of xenotropic murine leukemia virus-related virus by APOBEC3 proteins and antiviral drugs," *Journal of Virology*, vol. 84, no. 11, pp. 5719–5729, 2010.

[27] B. Oakes, A. K. Tai, O. Cingöz et al., "Contamination of human DNA samples with mouse DNA can lead to false detection of XMRV-like sequences," *Retrovirology*, vol. 7, no. 1, p. 109, 2010.

[28] M. J. Robinson, O. W. Erlwein, S. Kaye et al., "Mouse DNA contamination in human tissue tested for XMRV," *Retrovirology*, vol. 7, no. 1, p. 108, 2010.

[29] C. Qin, Z. Wang, J. Shang et al., "Intracisternal a particle genes: distribution in the mouse genome, active subtypes, and potential roles as species-specific mediators of susceptibility to cancer," *Molecular Carcinogenesis*, vol. 49, no. 1, pp. 54–67, 2010.

[30] K. Knox, D. Carrigan, G. Simmons et al., "No evidence of murine-like gammaretroviruses in CFS patients previously identified as XMRV-infected," *Science*, vol. 333, no. 6038, pp. 94–97, 2011.

[31] E. Sato, R. A. Furuta, and T. Miyazawa, "An endogenous murine leukemia viral genome contaminant in a commercial RT-PCR Kit is amplified using standard primers for XMRV," *Retrovirology*, vol. 7, no. 1, p. 110, 2010.

[32] P. W. Tuke, K. I. Tettmar, A. Tamuri, J. P. Stoye, and R. S. Tedder, "PCR master mixes harbour murine DNA sequences. Caveat emptor!," *PLoS ONE*, vol. 6, no. 5, article e19953, 2011.

[33] C. H. Shin, L. Bateman, R. Schlaberg et al., "Absence of XMRV retrovirus and other murine leukemia virus-related viruses in patients with chronic fatigue syndrome," *The Journal of Virology*, vol. 85, no. 14, pp. 7195–7202, 2011.

[34] J. P. Stoye, R. H. Silverman, C. A. Boucher, and S. F. J. Le Grice, "The xenotropic murine leukemia virus-related retrovirus debate continues at first international workshop," *Retrovirology*, vol. 7, no. 1, p. 113, 2010.

[35] C. Y. Thomas, R. Khiroya, R. S. Schwartz, and J. M. Coffin, "Role of recombinant ecotropic and polytropic viruses in the development of spontaneous thymic lymphomas in HRS/J mice," *Journal of Virology*, vol. 50, no. 2, pp. 397–407, 1984.

[36] J. P. Stoye, C. Moroni, and J. M. Coffin, "Virological events leading to spontaneous AKR thymomas," *Journal of Virology*, vol. 65, no. 3, pp. 1273–1285, 1991.

[37] A. S. Khan, "Nucleotide sequence analysis establishes the role of endogenous murine leukemia virus DNA segments in formation of recombinant mink cell focus-forming murine leukemia viruses," *Journal of Virology*, vol. 50, no. 3, pp. 864–871, 1984.

[38] S. Bhosle, S. Suppiah, R. Molinaro et al., "Evaluation of cellular determinants required for in vitro xenotropic murine leukemia virus-related virus entry into human prostate cancer and noncancerous cells," *Journal of Virology*, vol. 84, no. 13, pp. 6288–6296, 2010.

[39] R. A. Weiss, "A cautionary tale of virus and disease," *BMC Biology*, vol. 8, no. 1, p. 124, 2010.

[40] S. Hue, E. R. Gray, A. Gall et al., "Disease-associated XMRV sequences are consistent with laboratory contamination," *Retrovirology*, vol. 7, no. 1, p. 111, 2010.

[41] Y. Takeuchi, M. O. McClure, and M. Pizzato, "Identification of gammaretroviruses constitutively released from cell lines used for human immunodeficiency virus research," *Journal of Virology*, vol. 82, no. 24, pp. 12585–12588, 2008.

[42] K. P. Raisch, M. Pizzato, H. Y. Sun, Y. Takeuchi, L. W. Cashdollar, and S. E. Grossberg, "Molecular cloning, complete sequence, and biological characterization of a xenotropic murine leukemia virus constitutively released from the human B-lymphoblastoid cell line DG-75," *Virology*, vol. 308, no. 1, pp. 83–91, 2003.

[43] M. Pizzato, "MLV glycosylated-gag is an infectivity factor that rescues Nef-deficient HIV-1," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 20, pp. 9364–9369, 2010.

Hindawi Publishing Corporation
Advances in Virology
Volume 2011, Article ID 854540, 5 pages
doi:10.1155/2011/854540

Research Article

Failure to Detect XMRV-Specific Antibodies in the Plasma of CFS Patients Using Highly Sensitive Chemiluminescence Immunoassays

Brendan Oakes,[1,2] Xiaoxing Qiu,[3] Susan Levine,[4] John Hackett Jr.,[3] and Brigitte T. Huber[1]

[1] *Pathology Department, Tufts University School of Medicine, 150 Harrison Avenue, Boston, MA 02111, USA*
[2] *Pharmacology Program, Tufts University School of Medicine, 150 Harrison Avenue, Boston, MA 02111, USA*
[3] *Infectious Diseases R&D, Abbott Diagnostics, 100 Abbott Park Road, Abbott Park, IL 60064, USA*
[4] *Private Practice, 115 East 72nd Street, New York, NY 10021, USA*

Correspondence should be addressed to Brigitte T. Huber, brigitte.huber@tufts.edu

Received 8 April 2011; Accepted 10 June 2011

Academic Editor: Myra McClure

In 2009, Lombardi et al. reported their startling finding that the gammaretrovirus xenotropic murine leukemia virus-related retrovirus (XMRV) is present in 67% of blood samples of patients suffering from chronic fatigue syndrome (CFS), as opposed to only 3.7% of samples from healthy individuals. However, we and others could not confirm these results, using a nested PCR assay. An alternative to this highly sensitive, but contamination-prone, technique is to measure the serological response to XMRV. Thus, we tested the plasma samples from our cohorts of CFS patients and healthy controls for the presence of XMRV-specific antibodies. Using two novel chemiluminescence immunoassays (CMIAs), we show that none of our samples have any XMRV-reactive antibodies. Taken together with our previous findings, we conclude that XMRV is not present in any human individual tested by us, regardless of CFS or healthy control.

1. Introduction

In 2006, Urisman et al. identified a new gammaretrovirus in prostate cancer samples harboring a mutation in a viral defense gene known as *RNASEL* [1]. This new virus, xenotropic murine leukemia virus-related retrovirus (XMRV), was found to be a close relative to known murine leukemia viruses (MLVs) and was the first documented case of human infection with a xenotropic retrovirus. Although XMRV was originally associated with the mutant variant of the *RNASEL* gene, further research could not confirm this association but did find it in about 10% of prostate cancers [2].

The discovery of a new virus that could infect humans lead Lombardi et al. [3] to test for the virus in patients suffering from chronic fatigue syndrome (CFS). CFS is a disease of unknown etiology that manifests as neurological, immunological, and endocrinological dysfunctions. A wide range of viruses have been investigated in the past as causative agents of CFS; however, findings were mixed, and no conclusive evidence of one virus causing CFS has been implicated [4]. Using a nested polymerase chain reaction (PCR), Lombardi et al. found that blood samples of 68 out of 101 (67%) CFS patients contained the XMRV *gag* sequence, as opposed to only 8 out of 212 (3.7%) samples from healthy individuals [3]. The finding of a virus linked to CFS reignited excitement in the field, leading many laboratories around the world to test for this new virus, but the excitement has been short lived. Although some support linking XMRV or MLVs and CFS has been published [3, 5, 6], it has been overshadowed by reports failing to detect the virus in CFS patients [7–20], including a study done by us.

In our original paper [17], we failed to find an association between CFS patients and XMRV, using PCR technology. However, we did detect some XMRV sequences as well as other MLV sequences in some of our samples. Due to the close relationship between XMRV and MLVs, which are

present throughout the mouse genome, we tested all of our samples for mouse DNA using a TaqMan qPCR assay for murine mitochondrial cytochrome oxidase, *cox2* [14], as well as a single PCR assay for the highly abundant intracisternal A-type particle (IAP) long terminal repeat sequence, developed by our group [17]. We found that every sample that contained an XMRV or MLV sequence was also positive for mouse DNA contamination. Although we did not claim that our findings provided a full explanation of the origin of XMRV, we put forward a cautionary tale about the risks of mouse DNA contamination in various common laboratory reagents.

One of the criticisms of our study [17] was that we only used PCR technology to test for the presence of XMRV, while the original paper also included serological analyses [3]. Specifically, some groups have developed novel serological tests utilizing western blots and ELISAs in the search for anti-XMRV antibodies, because the presence of antibodies could not be due to mouse DNA contamination [3, 8, 13, 14, 20, 21]. Recently, two prototype direct format chemiluminescent immunoassays (CMIAs) were developed to detect XMRV-specific antibodies [22]. Both CMIAs utilize a direct assay format in which recombinant p15E or gp70 protein serves as both capture and detection antigens. The assays demonstrated excellent sensitivity, detecting early seroconversion bleeds in XMRV-infected rhesus macaques [22]. Moreover, these assays were also shown to detect specific antibodies to MLVs [22]. In this study, we use these two sensitive CMIAs to screen plasma samples from our blinded cohorts for the presence of XMRV-specific antibodies. No samples from our cohort of over 100 CFS patients were positive in either of these assays, while two samples from the healthy control cohort tested positive in one of the CMIA assays; however, reactivity of these same samples was not confirmed by western blot. Thus, these highly sensitive serological studies have confirmed our prior conclusion that the positive XMRV PCR results were a result of mouse DNA contamination, since no antibodies against XMRV were present.

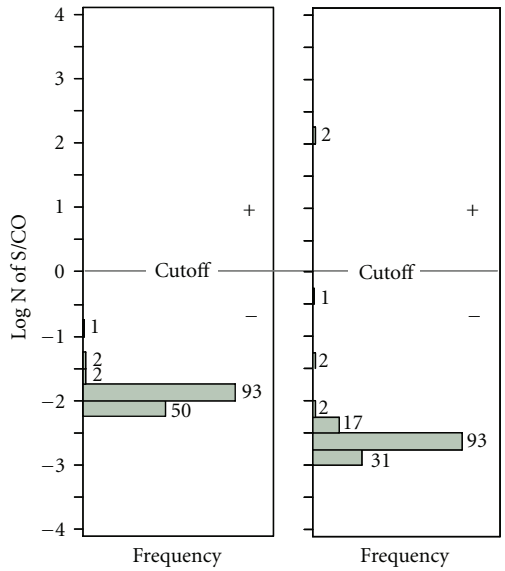
2. Materials and Methods

2.1. Sample Collection. All samples were collected according to the institutional guidelines of Tufts University, after receiving informed consent. The 36 healthy individuals (15 females and 21 males) were recruited on a voluntary basis by the Huber laboratory and were between 18 and 65 years of age. The 112 CFS patients (90 females, 20 males, and 3 unknown), recruited by Dr. Susan Levine, were between 18 and 65 years of age and resided in the Northeastern United States. All patients were diagnosed for CFS according to the CDC criteria, and the majority was completely disabled. The cohort comprised a combination of those with an abrupt and others with a gradual onset of symptoms.

2.2. Preparation of Human Blood and Plasma Samples. Approximately 30 mL of blood were drawn into three heparinized tubes (Becton Dickinson) and shipped overnight (CFS patients) or processed immediately (healthy controls). The blood collection tubes from each individual were consolidated into one 50 mL tube and diluted with PBS, containing CaCl2 and MgCl2 (sigma) at a 1 : 1 ratio. 15 mL of Ficoll (GE Healthcare) was added to two new 50 mL tubes, and 25 mL of the diluted blood was gently layered on top of the Ficoll, followed by a 30 min centrifugation in a Sorvall RT7 plus rotor at 2000 rpm at room temperature. The PBMCs were collected from the interface following the spin and were used for DNA isolation. Ten mL of plasma were also collected from each sample and stored at −80°C. One ml of plasma was sent to Abbott Labs on dry ice overnight for further testing.

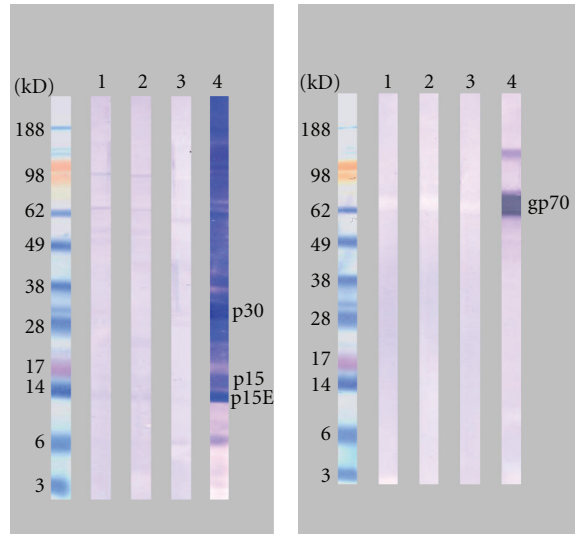
2.3. XMRV Chemiluminescent Immunoassays (CMIAs). A detailed procedure can be seen here [22]. Briefly, 100 μL of neat plasma were screened for antibodies to XMRV gp70 and p15E proteins using two prototype ARCHITECT chemiluminescent immunoassays (CMIAs; Abbott Diagnostics, Abbott Park, Ill). The CMIAs utilize a direct assay format in which *E. coli*-expressed XMRV p15E or mammalian-expressed XMRV gp70 were used as both capture and detection antigens. Assay positive controls were derived from XMRV-infected macaque plasmas at 1 : 1000 (PC1) or 1 : 4000 (PC2). A pool of normal human plasma was used as negative control (NC) and as sample diluents. Cutoff (CO) values of the ARCHITECT CMIAs were calculated based on the following formulas: CO = 0.45 × (Calibrator 1 Mean Relative Light Units (RLU)) for p15E CMIA and CO = 0.078 × (Calibrator 2 Mean RLU) for gp70 CMIA. Assay results were reported as the ratio of the sample RLU to the cutoff RLU (S/CO) for each specimen. Specimens with S/CO values <1.00 were considered nonreactive; specimens with S/CO values ≥1.00 were considered initially reactive. The S/CO values of the NC, PC1, and PC2 were 0.16, 12.8, and 3.5 for the gp70 CMIA and 0.13, 7.4, and 2.2 for the p15E CMIA. Initially reactive specimens were retested in duplicate by either ARCHITECT p15E or gp70 CMIAs. Repeatedly reactive specimens were analyzed at 1 : 100 dilution by investigational western blot assays using purified XMRV viral lysate as well as recombinant gp70 protein.

2.4. Western Blot Analysis. Western blot (WB) analysis using purified XMRV viral lysate as well as recombinant gp70 protein was performed as described [22]. Briefly, viral lysate (80 μg/gel) or recombinant gp70 protein (20 μg/gel) were separated by electrophoresis on a 4–12% NuPAGE Bis-Tris 2-dimension gel (Invitrogen, Carlsbad, Calif) in the presence of sodium dodecyl sulfate (SDS). The protein bands on the gel were electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane (invitrogen). After blocking, the PVDF membrane was cut into 2 mm strips. Strips were incubated with human samples diluted 1 : 100 or XMRV-infected macaque plasma diluted 1 : 200 overnight at 2–8°C. After removal of unbound antibodies, strips were incubated with alkaline phosphatase conjugated goat antihuman IgG (Southern Biotech, Birmingham, Ala) for 30 minutes at room temperature. The strips were washed, and chromogenic substrate solution was added.



(a) p15E CMIA (b) gp70 CMIA

Figure 1: Distribution of p15E CMIA (a) and gp70 CMIA (b) log N of S/CO on 148 samples collected from 112 CFS patients and 36 healthy controls. Numbers of specimens within each log N of S/CO value are shown above the solid bars. Assay cutoffs were equivalent to mean 16 SD and 12 SD for p15E and gp70 CMIAs, respectively, based on blood donor populations [22]. Log N of S/CO, natural log transformation of S/CO.



(a) XMRV Viral Lysate (b) Recombinant gp70

Figure 2: WB analysis of gp70 CMIA reactive samples with (a) native XMRV viral proteins and (b) recombinant gp70. WB strip key: 1 & 2: gp70 reactive samples 137 and 138; 3: normal blood donor plasma as negative control; 4: XMRV-infected macaque plasma as positive control. The faint white band in the 65–70 kd region in (B, strips no. 1–3) indicates a lack of specific anti-gp70 antibody.

3. Results

148 blinded plasma samples from our original CFS and healthy control cohorts were analyzed for the presence of XMRV-specific antibodies, using the direct format ARCHITECT p15E and gp70 CMIAs. None of the 148 plasma samples were reactive in the p15E CMIA (Figure 1(a)). Two of the 148 samples (ID = 137, 138) were positive in the gp70 CMIA (Figure 1(b)). Both specimens were weakly reactive in the gp70 CMIA with sample/cut-off (S/CO) values of 7.77 (log N of S/CO = 2.05) and 9.02 (log N of S/CO = 2.20), respectively. Although the samples were repeat reactive in the gp70 CMIA, they were not reactive by WB. As shown in Figure 2, both samples showed no visible WB bands using either XMRV viral lysate proteins (Figure 2(a)) or recombinant gp70 protein (Figure 2(b)). Unblinding of the samples revealed that the two gp70 reactive samples stemmed from two sequential blood collections of a single healthy control (Table 1).

4. Discussion

In our original study, we found no specific relationship between the presence of XMRV and CFS [17]. However, screening the genomic DNA from peripheral blood lymphocytes of both healthy control and CFS cohorts, we did detect PCR products that were identical to XMRV *gag* sequences, as well as other MLV *gag* sequences. Due to the high number of MLV sequences in the mouse genomic DNA, we found it prudent to test for mouse DNA contamination in our samples. Using both a test developed by the Switzer lab at CDC for mouse mitochondrial DNA [14], as well as a test developed by the Coffin lab for the IAP [17], we found that every sample that was positive for XMRV or other MLVs PCR products was also positive for mouse DNA. Although these data provide an explanation for the detection of MLV sequences in our samples, they do not rule out the possibility that XMRV and mouse DNA contamination could be present in the same sample. To clarify this issue, we tested our plasma samples for the presence of XMRV-specific antibodies.

Recent animal studies showed that XMRV infection elicited a potent humoral immune response in rhesus macaques [22]. The infected macaques developed XMRV-specific antibodies within two weeks of infection and persisted more than 158 days. The predominant responses were to all three structural proteins of XMRV: the envelope protein gp70, the transmembrane protein p15E, and the capsid protein p30 [22]. Sensitivity of both p15E and gp70 CMIAs was validated by the animal model; both CMIAs were able to detect p15E or gp70 specific antibodies as early as day 9 after infection [22]. In contrast, we were unable to detect XMRV p15E or gp70 specific antibodies in the 112 CFS patients and the 36 healthy controls. Although 2 samples from the same healthy control had weak reactivity in gp70 CMIA, the reactivity was not confirmed by recombinant gp70 WB. Furthermore, both samples were nonreactive in p15E CMIA and had no detectable p15E and p30 antibodies by viral lysate WB. Considered in combination with the negative PCR data, the observed isolated and weak gp70 reactivity

Table 1: Results summary for XMRV positive PCR samples. All samples that tested positive for XMRV *gag* sequence in original study [17], as well as the two samples that reacted with the gp70 CMIA, are displayed. Bolded samples showed the VP42 *gag* sequence but did not react with the CMIAs. The italic data shows the two samples that were reactive in the gp70 CMIA. CMIA values less than one are considered nonreactive. XMRV GAG: Nested gag PCR. Mcox: murine mitochondrial cytochrome oxidase qPCR. IAP: Intracisternal A-type particle PCR.

		PCR results			Initial test p15E	Initial test gp70	Repeat test gp70
ID	Unblinded ID	XMRV GAG	Mcox	IAP	S/CO	S/CO	S/CO
72	TH72.1	+	+	+	0.38	0.06	
128	TH04.1	+	+	+	0.16	0.07	
129	TH01.7	+	+	+	0.15	0.06	
131	TH01.8	+	−	+	0.12	0.06	
132	TH01.3	+	+	+	0.15	0.06	
134	**TH06.1**	**+**	**+**	**+**	**0.15**	**0.07**	
135	TH01.1	+	+	+	0.14	0.09	
136	TH05.1	+	+	+	0.16	0.06	
137	***TH07.1***	**+**	**+**	**+**	***0.16***	***7.77***	***7.17, 7.21***
138	***TH07.2***	**−**	**−**	**−**	***0.14***	***9.02***	***8.65, 8.77***
143	TH10.1	+	+	+	0.14	0.07	
144	TH11.1	+	−	+	0.14	0.06	
147	TH02.1	+	+	+	0.14	0.07	
152	TH01.5	+	+	+	0.13	0.07	
153	TH21.1	+	+	+	0.15	0.07	
155	TH20.1	+	+	+	0.16	0.06	
156	TH02.2	+	+	+	0.17	0.07	
158	**TH08.1**	**+**	**+**	**+**	**0.13**	**0.07**	
160	**TH03.1**	**+**	**+**	**+**	**0.13**	**0.07**	
161	TH12.1	+	+	+	0.11	0.06	
163	TH19.1	+	+	+	0.16	0.72	0.75, 0.72
164	TH16.1	+	+	+	0.15	0.07	

most likely represents nonspecific reactivity since specificity of the gp70 CMIA was reported as 99.5% [22]. In summary, the serologic data obtained in this study suggests a lack of XMRV infection in our CFS patients and healthy controls. It is theoretically possible that XMRV replicates at very low levels in humans and fails to induce a humoral immune response, or, alternatively, that it is sequestered or latent and specific antibody titers have declined to undetectable levels over time. Although these possibilities cannot be formally excluded, they seem unlikely given responses observed to other human retroviruses. The combination of negative molecular and serologic data do not support an association between CFS and XMRV or other MLVs. Furthermore, the recent demonstration that XMRV is a recombinant of two murine MLVs (23) raises doubts about the validity (24) of the original XMRV claims in CFS (3).

5. Conclusion

With the serological data added to our original finding, we can unequivocally conclude that XMRV is not present in our CFS patient or healthy control cohort samples. Although we have detected XMRV *gag* sequences in three of our samples, they all tested positive for mouse DNA and tested negative for XMRV-specific antibodies. Laboratory mouse strains, as well as wild mice, all carry numerous endogenous MLVs, and extreme caution must be taken when testing for murine-related viruses.

References

[1] A. Urisman, R. J. Molinaro, N. Fischer et al., "Identification of a novel gammaretrovirus in prostate tumors of patients homozygous for R462Q RNASEL variant," *PLoS Pathogens*, vol. 2, no. 3, Article ID e25, 2006.

[2] R. Schlaberg, D. J. Choe, K. R. Brown, H. M. Thaker, and I. R. Singh, "XMRV is present in malignant prostatic epithelium and is associated with prostate cancer, especially high-grade tumors," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 38, pp. 16351–16356, 2009.

[3] V. C. Lombardi, F. W. Ruscetti, J. D. Gupta et al., "Detection of an infectious retrovirus, XMRV, in blood cells of patients with chronic fatigue syndrome," *Science*, vol. 326, no. 5952, pp. 585–589, 2009.

[4] B. M. Carruthers, A. K. Jain, K. L. De Meirleir et al., "Myalgic encephalomyelitis/chronic fatigue syndrome: clinical working

case definition, diagnostic and treatment protocols," *Journal of Chronic Fatigue Syndrome*, vol. 11, no. 1, pp. 7–115, 2003.

[5] J. A. Mikovits, V. C. Lombardi, M. A. Pfost, K. S. Hagen, and F. W. Ruscetti, "Detection of an infectious retrovirus, XMRV, in blood cells of patients with chronic fatigue syndrome," *Virulence*, vol. 1, no. 5, pp. 386–390, 2010.

[6] S. C. Lo, N. Pripuzova, B. Li et al., "Detection of MLV-related virus gene sequences in blood of patients with chronic fatigue syndrome and healthy blood donors," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 36, pp. 15874–15879, 2010.

[7] O. Erlwein, S. Kaye, M. O. McClure et al., "Failure to detect the novel retrovirus XMRV in chronic fatigue syndrome," *PLoS ONE*, vol. 5, no. 1, Article ID e8519, 2010.

[8] H. C. T. Groom, V. C. Boucherit, K. Makinson et al., "Absence of xenotropic murine leukaemia virus-related virus in UK patients with chronic fatigue syndrome," *Retrovirology*, vol. 7, article no. 10, 2010.

[9] F. J. van Kuppeveld, A. S. de Jong, K. H. Lanke et al., "Prevalence of xenotropic murine leukaemia virus-related virus in patients with chronic fatigue syndrome in the Netherlands: retrospective analysis of samples from an established cohort," *British Medical Journal*, vol. 340, Article ID c1018, 2010.

[10] W. M. Switzer, H. Jia, O. Hohn et al., "Absence of evidence of Xenotropic Murine Leukemia Virus-related virus infection in persons with Chronic Fatigue Syndrome and healthy controls in the United States," *Retrovirology*, vol. 7, article no. 57, 2010.

[11] P. Hong, J. Li, and Y. Li, "Failure to detect Xenotropic murine leukaemia virus-related virus in Chinese patients with chronic fatigue syndrome," *Virology Journal*, vol. 7, article no. 224, 2010.

[12] T. J. Henrich, J. Z. Li, D. Felsenstein et al., "Xenotropic murine leukemia virus-related virus prevalence in patients with chronic fatigue syndrome or chronic immunomodulatory conditions," *Journal of Infectious Diseases*, vol. 202, no. 10, pp. 1478–1481, 2010.

[13] O. Hohn, K. Strohschein, A. U. Brandt et al., "No evidence for XMRV in German CFS and MS patients with fatigue despite the ability of the virus to infect human blood cells *in vitro*," *PLoS ONE*, vol. 5, no. 12, Article ID e15632, 2010.

[14] B. C. Satterfield, R. A. Garcia, H. Jia et al., "Serologic and PCR testing of persons with chronic fatigue syndrome in the United States shows no Association with xenotropic or polytropic murine leukemia virus-related viruses," *Retrovirology*, vol. 8, article no. 12, 2011.

[15] O. Erlwein, M. J. Robinson, S. Kaye et al., "Investigation into the presence of and serological response to XMRV in CFS patients," *PLoS ONE*, vol. 6, no. 3, Article ID e17592, 2011.

[16] M. J. Robinson, O. W. Erlwein, S. Kaye et al., "Mouse DNA contamination in human tissue tested for XMRV," *Retrovirology*, vol. 7, article no. 108, 2010.

[17] B. Oakes, A. K. Tai, O. Cingoz et al., "Contamination of human DNA samples with mouse DNA can lead to false detection of XMRV-like sequences," *Retrovirology*, vol. 7, article no. 109, 2010.

[18] E. Sato, R. A. Furuta, and T. Miyazawa, "An endogenous murine leukemia viral genome contaminant in a commercial RT-PCR Kit is amplified using standard primers for XMRV," *Retrovirology*, vol. 7, article no. 110, 2010.

[19] S. Hue, E. R. Gray, A. Gall et al., "Disease-associated XMRV sequences are consistent with laboratory contamination," *Retrovirology*, vol. 7, article no. 111, 2010.

[20] R. A. Furuta, T. Miyazawa, T. Sugiyama et al., "No association of xenotropic murine leukemia virus-related virus with prostate cancer or chronic fatigue syndrome in Japan," *Retrovirology*, vol. 8, article no. 20, 2011.

[21] R. S. Arnold, N. V. Makarova, A. O. Osunkoya et al., "XMRV infection in patients withprostate cancer: novel serologic assay and correlation with PCR and FISH," *Urology*, vol. 75, no. 4, pp. 755–761, 2010.

[22] X. Qiu, P. Swanson, K. C. Luk et al., "Characterization of antibodies elicited by XMRV infection and development of immunoassays useful for epidemiologic studies," *Retrovirology*, vol. 7, article no. 68, 2010.

Hindawi Publishing Corporation
Advances in Virology
Volume 2011, Article ID 268214, 6 pages
doi:10.1155/2011/268214

Research Article

Prevalence of XMRV Nucleic Acid and Antibody in HIV-1-Infected Men and in Men at Risk for HIV-1 Infection

J. Spindler,[1] J. Hackett Jr.,[2] X. Qiu,[2] A. Wiegand,[1] V. F. Boltz,[1] P. Swanson,[2] J. H. Bream,[3] L. P. Jacobson,[3] X. Li,[3] C. R. Rinaldo,[4] S. M. Wolinsky,[5] J. M. Coffin,[6] M. F. Kearney,[1] and J. W. Mellors[4]

[1] *HIV Drug Resistance Program, National Cancer Institute, Frederick, MD 21702-1201, USA*
[2] *AIDS Research and Retrovirus Discovery, Abbott Laboratories, Abbott Park, IL 60064, USA*
[3] *Bloomberg School of Public Health, Johns Hopkins University, Baltimore, MD 21205, USA*
[4] *Department of Medicine, University of Pittsburgh, Pittsburgh, PA 15261, USA*
[5] *Feinberg School of Medicine, Northwestern University Chicago, IL 60611, USA*
[6] *Department of Molecular Biology and Microbiology, Tufts University, Boston, MA 02111, USA*

Correspondence should be addressed to M. F. Kearney, kearneym@mail.nih.gov

Received 20 June 2011; Revised 8 August 2011; Accepted 22 August 2011

Academic Editor: Arifa S. Khan

Xenotropic MLV-Related Virus (XMRV) was recently reported to be associated with prostate cancer and chronic fatigue syndrome (CFS). Infection was also reported in 3.7% of healthy individuals. These highly reported frequencies of infection prompted concerns about the possibility of a new, widespread retroviral epidemic. The Multicenter AIDS Cohort Study (MACS) provides an opportunity to assess the prevalence of XMRV infection and its association with HIV-1 infection among men who have sex with men. Reliable detection of XMRV infection requires the application of multiple diagnostic methods, including detection of human antibodies to XMRV and detection of XMRV nucleic acid. We, therefore, tested 332 patient plasma and PBMC samples obtained from recent visits in a subset of patients in the MACS cohort for XMRV antibodies using Abbott prototype ARCHITECT chemiluminescent immunoassays (CMIAs) and for XMRV RNA and proviral DNA using a XMRV single-copy qPCR assay (X-SCA). Although 9 of 332 (2.7%) samples showed low positive reactivity against a single antigen in the CMIA, none of these samples or matched controls were positive for plasma XMRV RNA or PBMC XMRV DNA by X-SCA. Thus, we found no evidence of XMRV infection among men in the MACS regardless of HIV-1 serostatus.

1. Introduction

Xenotropic Murine Leukemia Virus-Related Virus (XMRV) is a recently discovered gammaretrovirus reportedly associated with prostate cancer and chronic fatigue syndrome (CFS) [1, 2]. Urisman et al. first identified XMRV in 2006 in a cohort of prostate cancer patients [2], followed by Lombardi et al. who reported XMRV infection in 67% of patients with severe CFS and 3.7% of healthy individuals [1]. These initial reports provided a compelling rationale for further investigations into the prevalence of XMRV infection in human populations. However, controversy arose when subsequent studies failed to detect the virus in similar cohorts [3–7]. It was suggested that inconsistencies in detection of XMRV in patient samples could result from varied incidence of infection in different populations, differing criteria for patient selection, and differing detection methods [8]. It was also proposed that virus levels may be chronically low or episodic in patient plasma or tissues, making virus detection difficult [8]. Adding to the complexity, detection of XMRV by PCR is highly susceptible to false positive results due to amplification of closely related endogenous Murine Leukemia Viruses (MLVs) in the mouse genome and the high prevalence of contaminating mouse genomic DNA in many specimens and reagents [9, 10]. Additionally, studies have suggested that XMRV detection is the result of laboratory contamination from infected cell lines [11–14]. Paprotka et al. proposed that XMRV originated as a laboratory artifact when two endogenous mouse proviruses recombined during passaging of a human prostate cancer tumor in nude mice, an event that

is highly unlikely to have occurred more than once. The authors, therefore, concluded that published XMRV sequences obtained from patient samples must have come from contamination of samples by virus or DNA from cell lines infected with this recombinant virus [14]. To investigate the human prevalence of XMRV infection, it is clear that reliable detection requires the application of several diagnostic methods used together, including methods that are not influenced by nucleic acid contamination, to avoid reporting potentially high rates of false positives.

Accordingly, we analyzed recently collected blood samples from participants in the MACS cohort using new tests that detect XMRV antibodies and nucleic acid in the blood stream [15]. The MACS cohort provided the opportunity to assess the association of XMRV with HIV-1 infection and other clinical outcomes and to evaluate its possible mode of transmission. We hypothesized that the prevalence of XMRV infection is higher among men who have acquired HIV-1 infection than among seronegative controls. Previous studies have evaluated samples from HIV-infected cohorts for the presence of XMRV nucleic acid with negative results [7, 16, 17], but none has looked for the presence of antibody to XMRV.

In the current study, we first screened samples for antibody reactivity to XMRV. This approach eliminated the risk that positive results were due to nucleic acid contamination and mitigated the risk that infection would be missed due to low-level or episodic viremia. To further minimize the risk of reporting false-positive XMRV infection status, we required that antibody and nucleic acid (either viral RNA or DNA) must both be present to report the patient as being XMRV infected. These criteria are supported by studies performed on XMRV-inoculated macaques confirming that both antibody and nucleic acid are readily detectable in longitudinal blood samples collected after XMRV infection (Kearney et al. in press; Del Prete et al. in preparation) [15, 18]. Plasma samples from the MACS cohort were screened for antibody reactivity by CMIAs and confirmed by Western blot. Reactive samples and blinded, matched antibody negative controls from the same cohort were then tested for the presence of XMRV RNA in plasma and proviral DNA in PBMCs. This approach to determine the prevalence of XMRV infection minimizes the risk of reporting false positives that occur from either cross-reactive antibodies in the bloodstream or contaminating nucleic acid in samples or reagents.

2. Methods

2.1. Clinical Samples. Plasma and PBMC samples were obtained from 332 individuals in the MACS cohort. All samples were collected from clinical visits in 2006–2009. Half of the 332 men were HIV-1 seropositive, of whom 89 were users of antiretroviral therapy (ART) and 77 were ART naïve. HIV-1-seronegative men of similar age, date of study entry, center, and hepatitis status were selected from the same visits as the HIV-1-seropositive men. The median age (interquartiles (25%, 75%)) of both the HIV-1-seropositive and -seronegative men was 50 years (45, 55.5), and the median (IQR) CD4 cell count and HIV-1 RNA levels of the HIV-1 seropositives were 578 (418, 786) cells/mm^3 and <50 (<50, 12408) copies/mL, respectively. Plasma samples were screened at Abbott Laboratories for the presence of XMRV antibodies by newly developed XMRV CMIAs (ARCHITECT platform) [15]. Samples with positive CMIA results were tested by Western blot [15]. Samples that were positive for XMRV antibodies by CMIA were matched 1 : 3 by age, HIV serostatus, antiretroviral therapy, and pre-ART CD4 cell count for those who had initiated therapy, with CMIA negative samples. All samples were then assayed blinded for the presence of XMRV plasma RNA and proviral DNA in PBMCs using highly sensitive XMRV qPCR assays (X-SCA) (Kearney et al. submitted in press).

2.2. XMRV Chemiluminescent Immunoassays (CMIAs) and Western Blot. A detailed procedure has been described previously [15]. Briefly, 100 μL of neat plasma were screened for antibodies to XMRV gp70 (SU) and p15E (TM) proteins using two prototype ARCHITECT chemiluminescent immunoassays (CMIAs; Abbott Diagnostics, Abbott Park, IL). The CMIAs utilize a direct assay format in which *E. coli*-expressed XMRV p15E or mammalian-expressed XMRV gp70 was used as both capture and detection antigens. Assay positive controls were derived from XMRV-infected rhesus macaque plasma at 1 : 1000 (PC1) or 1 : 4000 (PC2). A pool of normal human plasma was used as negative control (NC). Cutoff (CO) values of the ARCHITECT CMIAs were calculated based on the following formulas: CO = 0.45 × (PC2 Mean Relative Light Units (RLU)) for p15E CMIA and CO = 0.078 × (PC1 Mean RLU) for gp70 CMIA. Assay results were reported as the ratio of the sample RLU to the cutoff RLU (S/CO) for each specimen. Specimens with S/CO values <1.00 were considered nonreactive; specimens with S/CO values >1.00 were considered initially reactive. Reactive specimens were further analyzed by ARCHITECT p30 CMIA and by investigational western blot assays.

ARCHITECT p30 CMIA also utilizes the direct assay format with *E. coli*-expressed XMRV p30 (capsid protein) to capture and detect anti-p30 antibodies [15]. The same sample volume (100 μL), calibrator, and controls were used for the p30 CMIA. CO of p30 CMIA was calculated based on the formula: CO = 0.27 × (PC1 Mean RLU).

Western blot (WB) analysis using purified XMRV viral lysate as well as recombinant gp70 protein was performed as described [15]. Briefly, viral lysate (65 μg/gel) or recombinant gp70 protein (25 μg/gel) was separated by electrophoresis on a 4–12% NuPAGE Bis-Tris 2-dimension gel (Invitrogen, Carlsbad, CA) in the presence of sodium dodecylsulfate (SDS). The protein bands on the gel were electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane (Invitrogen). After blocking, the PVDF membrane was cut into 2 mm strips. Strips were incubated with human plasma samples diluted 1 : 100 or XMRV-infected macaque plasma [18] diluted 1 : 250 overnight at 2–8°C. After removal of unbound antibodies, strips were incubated with alkaline phosphatase conjugated goat antihuman IgG (Southern Biotech, Birmingham, AL) for 30 minutes at room temperature.

The strips were washed, and chromogenic substrate solution was added to visualize the reactive bands.

2.3. Nucleic Assay Detection with XMRV Single-Copy Assays (X-SCA). Similar to the HIV-1 single-copy assay (SCA) [19], real-time PCR and RT-PCR assays for detection of XMRV, called X-SCA (XMRV single-copy assay), were developed to quantify XMRV RNA in plasma and proviral DNA in PBMCs (Kearney et al. in press). X-SCA was designed using amplification primers targeting a conserved *gag* leader region between XMRV and endogenous MLVs allowing detection of both templates. The TaqMan probe was designed to span the signature 15–24 nt deletion (derived from PreXMRV-2 [14] in the XMRV gag leader region compared to other MLV sequences). This probe design results in a lower plateau level of fluorescence from non-XMRV templates than from XMRV templates, thus distinguishing the source of product that is being detected in the assay (Kearney et al. in press). To confirm any positive result, PCR products are run on a 2% agarose gel, allowing distinction between the 86 nt XMRV X-SCA product and the 110 nt non-XMRV product.

Patient samples from the MACs cohort were tested by X-SCA in triplicate with equal numbers of no template controls (NTC) to monitor the level of false positives due to common mouse genomic DNA contamination. An internal RCAS (avian retrovirus) control was spiked into each plasma sample to quantify nucleic acid recovery from plasma samples as described previously [19]. X-SCA was performed on 0.1-0.2 mL of plasma resulting in a limit of detection of 9–18 RNA copies/mL and on 1×10^6 PBMCs resulting in a limit of detection of 1 DNA copy/1×10^6 cells.

2.4. Criteria for Determining XMRV Infection Status. There is a high risk of obtaining false positive results from CMIAs and PCR diagnostic tests [9, 10]. Therefore, we set strict criteria for declaring a sample positive for XMRV. We required that the sample must test positive for both XMRV antibody by CMIAs or Western blot and nucleic acid (either RNA or DNA) by X-SCA. A positive X-SCA test required detection of virus in all triplicate PCR reactions. If discordant results were obtained from triplicate wells, then the result was considered indeterminate.

2.5. Data Analysis and Sample Size Calculations. The sample sizes of 200 HIV-1-seropositive and 200 seronegative participants initially were chosen with arcsine approximation to provide 86% power to detect a difference in XMRV prevalence assuming 12% XMRV prevalence in HIV-1 seropositives versus 4% in seronegatives, with $\alpha = 0.05$. The 4% rate among HIV-1 seronegatives corresponded to the 3.7% prevalence observed in published control series [1]. Selection of HIV-1-seropositive men in the current ART era limited the inclusion of ART-naïve men, and the need for unthawed PBMC pellets and EDTA plasma further reduced the sample size to 332. Given the small number of positive results, simple frequencies are used to describe the data.

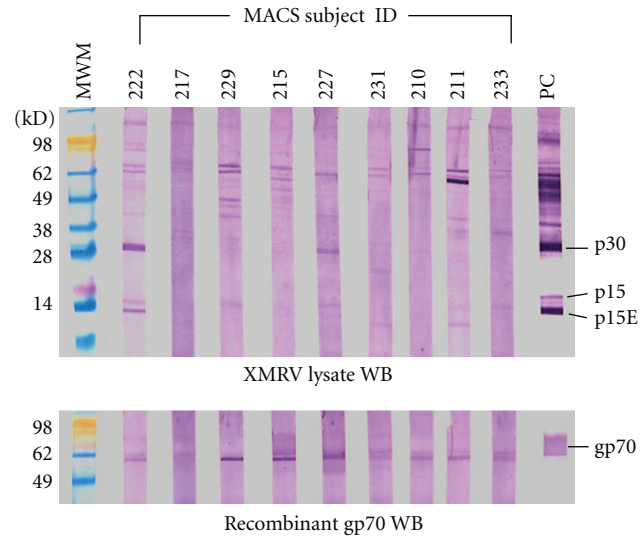


Figure 1: *XMRV Western Blot Analysis.* WB analysis of CMIA reactive MACS subjects using native XMRV viral lysate and recombinant gp70 protein. XMRV-infected macaque plasma was used as positive control (PC). Sample 222 tested positive for p15E by CMIA; all the others were positive for gp70 (Table 1).

3. Results

3.1. XMRV Serology with ARCHITECT CMIAs and Western Blot. Using the direct format ARCHITECT p15E and gp70 CMIAs, XMRV serology was evaluated on 332 recently collected plasma samples from patients in the MACS cohort. Nine samples (5 HIV-1 seropositive, 4 HIV-1 seronegative) were found to be reactive to XMRV proteins, one against the p15E transmembrane protein and 8 against the gp70 envelope protein (Table 1), resulting in a frequency of 0.3% (1/332) positive for p15E and 2.4% (8/332) for gp70. Of note, however, none of the samples were reactive against both p15E and gp70. Subsequent testing of the 9 positive samples with the p30 CMIA showed no detectable anti-p30 antibodies (Table 1).

By viral lysate WB, the only p15E CMIA-positive sample 222 was reactive to native p15E protein (Table 1, Figure 1). However, only 1 of the 8 gp70 CMIA-positive samples (215) was reactive in the recombinant gp70 WB. The low confirmation rate could be due to higher sensitivity of the gp70 CMIA or the antibodies detected may primarily recognize conformational epitopes which were sensitive to SDS or thermal denaturation. Although nonreactive in p30 CMIA, two samples, 222 and 227 had an apparent p30 band in the viral lysate WB (Table 1, Figure 1). However, subsequent WB analysis of CMIA-negative normal blood donors showed the presence of p30 band indicative of nonspecificity or cross-reactivity (data not shown). Others have also observed the nonspecific reactivity against XMRV p30 protein in WB [20].

3.2. Nucleic Assay Detection with XMRV Single-Copy Assays (X-SCA). XMRV single-copy assays (X-SCA) were conducted on both plasma and PBMCs from the 9 patients with CMIA-reactive antibodies, and 25 CMIA-negative controls

Table 1: XMRV antibody reactivity for the MACS Subjects.

MACS ID	CMIA (S/CO)			WB
	p15E TM	gp70 SU	p30* CA	
222	*1.67*	0.08	0.23	p15E+, p30+
217	0.22	*28.48*	0.17	
229	0.18	*8.73*	0.19	
215	0.17	*21.36*	0.21	gp70+
227	0.20	*2.27*	0.18	p30+
231	0.17	*1.56*	0.18	
210	0.18	*1.65*	0.22	
211	0.17	*1.47*	0.20	
233	0.19	*1.80*	0.20	
Control				
NC	0.17	0.11	0.23	
PC1	*7.90*	*12.82*	*3.70*	gp70+, p15E+, p30+
PC2	*2.22*	*3.47*	*1.10*	NA

* Samples were tested at 1 : 2.5–5 dilutions due to limited sample volume.

matched for date of MACS entry, HIV serostatus, ART use, time from ART initiation, and pre-ART CD4+ T-cell count (Table 2). None of the samples tested were found to be positive for XMRV nucleic acid by X-SCA (either RNA or DNA) including the 9 samples that were CMIA reactive. Given the sample volumes available for testing, X-SCA had limits of detection for XMRV RNA in plasma of 9.2 copies/mL and 1 XMRV DNA copy in one million PBMCs. Sixty-one copies of RNA were detected in one of triplicates from sample 218, resulting in an indeterminate X-SCA result for this subject. The same sample was CMIA nonreactive, and no XMRV DNA was detected in one million PBMCs. Consequently, the sample did not meet our criteria for a positive XMRV result. A single positive well in an X-SCA run is not above the level of false positives for XMRV real-time PCR assays due to the high frequency of environmental mouse genomic DNA that readily amplifies with the X-SCA primers (Kearney et al. in press).

4. Discussion

The XMRV study by Lombardi et al. published in October 2009 suggested a surprisingly high seroprevalence for XMRV, even among healthy control subjects [1]. Therefore, we set out to evaluate the prevalence of XMRV infection in a possible high-risk cohort. We adopted a multiple diagnostic assay approach to determine the XMRV status of patient samples to minimize the possibility of obtaining false positive results by individual molecular or serologic methods. False positive PCR results may occur from incidental amplification of environmental mouse genomic DNA due to the close relationship between XMRV and mouse proviruses [9, 10]. False positive serology results may occur due to nonspecific or cross-reactive human antibodies [15]. In fact, a recent study reported that ~4% of HTLV-I-infected individuals had antibodies that were cross-reactive to XMRV p15E protein [21]. Therefore, we applied stringent criteria for XMRV positivity aimed at limiting the risk of reporting false positive results. Our criteria for an XMRV infection required that (1) all replicates from X-SCA must be positive, (2) antibodies must be detectable by CMIA and/or Western blot, and (3) nucleic acid and antibody must both be positive. Samples resulting in discordant results from X-SCA replicates were reported as indeterminate. Applying these criteria, we did not identify any samples from the MACS cohort that were consistent with XMRV infection. Nine samples had positive serology, but no detectable XMRV nucleic acid. Although one sample was indeterminate for XMRV RNA by X-SCA, it was antibody negative. XMRV DNA was not detected in any sample tested. Because we did not find any patient to be reactive to multiple XMRV antigens and because all nine patients for whom serum reactivity was observed were negative for XMRV nucleic acid, we concluded that follow-up testing by additional methods, such as virus culture, was not necessary. We believe that the combined approach of sensitive antibody screening and sensitive and specific nucleic acid testing excludes XMRV infection in the cohort studied.

Previous studies have shown that X-SCA is able to detect XMRV RNA and DNA in spiked control samples and in specimens from inoculated macaques with high sensitivity [22] (Kearney et al. in press; Del Prete et al. in preparation). In the samples analyzed here, the sensitivity for detection was limited by the small sample volumes available for testing. Despite this, the limits of detection were still adequate to detect XMRV DNA that has been shown to persist in macaque PBMC samples following XMRV infection (Del Prete et al. in preparation). Consequently, proviral DNA would likely have been detectable in any subject recently infected with XMRV. It has been argued that XMRV nucleic acid could be missed in clinical samples from infected individuals due to low-level or episodic viremia. By testing a large cohort of HIV-infected individuals for immunity to XMRV and not for viral nucleic acid alone, we reduced the risk of reporting false negatives for XMRV infection.

Despite earlier reports that evidence of XMRV infection was detected in about 20% of prostate cancer patients [2, 23–25] and 67% of CFS patients [1], we did not find clear evidence for XMRV infection in a cohort of men with HIV-1 infection or at high risk for HIV-1 infection. These results are consistent with prior reports that failed to detect XMRV nucleic acid in HIV-1-infected patients [7, 16, 17, 26].

Findings from previous studies reporting higher prevalence for XMRV infection in cohorts typically involved testing by a single diagnostic method. In the current study, if we had based XMRV infection on a single diagnostic method (either PCR or serology), the apparent XMRV prevalence would have been 1.2%; 0.3% (1/332) by PCR and 0.9% (3/332) by serology. Given the potential for false positive results in PCR and serological assays for XMRV, our results suggest that applying multiple diagnostic methods including measuring levels of proviral DNA in blood cells provides a more reliable approach for investigating the prevalence of XMRV infection. We hypothesized that the prevalence of XMRV infection would be higher among men who have acquired HIV-1 infection than among seronegative controls.

Table 2: XMRV nucleic acid and antibody results.

Subject ID	XMRV average RNA copies/mL by X-SCA	XMRV DNA copies per 1e6 cells by X-SCA	ARCHITECT antibody serology	Western blot	XMRV status
201	<10	<1	neg	NT	neg
202	<10	<1	neg	NT	neg
203	<10	<1	neg	NT	neg
204	<9	<1	neg	NT	neg
205	<18	<1	neg	NT	neg
206	<9	<1	neg	NT	neg
207	<9	<1	neg	NT	neg
208	<9	<1	neg	NT	neg
209	<9	<1	neg	NT	neg
210	<9	<1	*gp70+*	*neg*	neg
211	<9	<1	*gp70+*	*neg*	neg
212	<9	<1	neg	NT	neg
213	<9	<1	neg	NT	neg
214	<9	<1	neg	NT	neg
215	<18	<1	*gp70+*	*gp70+*	neg
216	<9	<1	neg	NT	neg
217	<9	<1	*gp70+*	*neg*	neg
218	*61*	<1	neg	NT	neg
219	<9	<1	neg	NT	neg
220	<9	<1	neg	NT	neg
221	<9	<1	neg	NT	neg
222	<9	<1	*p15E+*	*p15E+, p30+*	neg
223	<9	<1	neg	NT	neg
224	<9	<1	neg	NT	neg
225	<9	<1	neg	NT	neg
226	<9	<1	neg	NT	neg
227	<9	<1	*gp70+*	*p30+*	neg
228	<9	<1	neg	NT	neg
229	<9	<1	*gp70+*	*neg*	neg
230	<9	<1	neg	NT	neg
231	<9	<1	*gp70+*	*neg*	neg
232	<9	<1	neg	NT	neg
233	<9	<1	*gp70+*	*neg*	neg
234	<9	<1	neg	NT	neg

* NT indicates that MACS ID was not tested by Western blot assay.

The negative data from our study clearly refute this hypothesis. Individuals at risk for HIV-1 infection and sexually transmitted infections are not at risk for XMRV infection.

Acknowledgments

The authors thank Frank Maldarelli and Stuart Legrice for helpful discussions and Connie Kinna, Susan Jordan, and Susan Toms for administrative support. Funding for this research was provided by the National Cancer Institute's intramural Center for Cancer Research and Office for AIDS Research and by an extramural contract from the National Cancer Institute (SAIC 25XS119) to the University of Pittsburgh (JWM). J. M. Coffin was a Research Professor of the American Cancer Society with support from the FM Kirby Foundation. Data in this paper were collected by the Multicenter AIDS Cohort Study (MACS) with centers (Principal Investigators) at the Johns Hopkins Bloomberg School of Public Health (Joseph B. Margolick, Lisa P. Jacobson), Howard Brown Health Center, Feinberg School of Medicine, Northwestern University, and Cook County Bureau of Health Services (John P. Phair, Steven M. Wolinsky), University of California, Los Angeles (Roger Detels), and University of Pittsburgh (Charles R. Rinaldo). The MACS is funded by the National Institute of Allergy and Infectious Diseases, with

additional supplemental funding from the National Cancer Institute: UO1-AI-35042, UL1-RR025005 (GCRC), UO1-AI-35043, UO1-AI-35039, UO1-AI-35040, UO1-AI-35041. Website located at http://www.statepi.jhsph.edu/macs/macs.html. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the US government.

References

[1] V. C. Lombardi, F. W. Ruscetti, J. Das Gupta et al., "Detection of an infectious retrovirus, XMRV, in blood cells of patients with chronic fatigue syndrome," *Science*, vol. 326, no. 5952, pp. 585–589, 2009.

[2] A. Urisman, R. J. Molinaro, N. Fischer et al., "Identification of a novel gammaretrovirus in prostate tumors of patients homozygous for R462Q RNASEL variant," *PLoS Pathogens*, vol. 2, article e25, 2006.

[3] B. C. Satterfield, R. A. Garcia, H. Jia, S. Tang, H. Zheng, and W. M. Switzer, "Serologic and PCR testing of persons with chronic fatigue syndrome in the United States shows no association with xenotropic or polytropic murine leukemia virus-related viruses," *Retrovirology*, vol. 8, article 12, 2011.

[4] O. Hohn, K. Strohschein, A. U. Brandt et al., "No evidence for XMRV in German CFS and MS patients with fatigue despite the ability of the virus to infect human blood cells in vitro," *PLoS One*, vol. 5, no. 12, Article ID e15632, 2010.

[5] M. Cornelissen, F. Zorgdrager, P. Blom et al., "Lack of detection of XMRV in seminal plasma from HIV-1 infected men in The Netherlands," *PLoS One*, vol. 5, no. 8, Article ID e12040, 2010.

[6] O. Erlwein, S. Kaye, M. O. McClure et al., "Failure to detect the novel retrovirus XMRV in chronic fatigue syndrome," *PLoS One*, vol. 5, no. 1, Article ID e8519, 2010.

[7] E. R. Gray, J. A. Garson, J. Breuer et al., "No evidence of XMRV or related retroviruses in a london HIV-1-positive patient cohort," *PLoS One*, vol. 6, no. 3, Article ID e18096, 2011.

[8] M. Kearney and F. Maldarelli, "Current status of xenotropic murine leukemia virus-related retrovirus in chronic fatigue syndrome and prostate cancer: reach for a scorecard, not a prescription pad," *Journal of Infectious Diseases*, vol. 202, no. 10, pp. 1463–1466, 2010.

[9] B. Oakes, A. K. Tai, O. Cingoz et al., "Contamination of human DNA samples with mouse DNA can lead to false detection of XMRV-like sequences," *Retrovirology*, vol. 7, article 109, 2010.

[10] M. J. Robinson, O. W. Erlwein, S. Kaye et al., "Mouse DNA contamination in human tissue tested for XMRV," *Retrovirology*, vol. 7, article 108, 2010.

[11] J. A. Garson, P. Kellam, and G. J. Towers, "Analysis of XMRV integration sites from human prostate cancer tissues suggests PCR contamination rather than genuine human infection," *Retrovirology*, vol. 8, article 13, 2011.

[12] S. Hué, E. R. Gray, A. Gall et al., "Disease-associated XMRV sequences are consistent with laboratory contamination," *Retrovirology*, vol. 7, article 111, 2010.

[13] K. Knox, D. Carrigan, G. Simmons et al., "No evidence of murine-like gammaretroviruses in CFS patients previously identified as XMRV-infected," *Science*, vol. 333, no. 6038, pp. 94–97, 2011.

[14] T. Paprotka, K. A. Delviks-Frankenberry, O. Cingöz et al., "Recombinant origin of the retrovirus XMRV," *Science*, vol. 333, no. 6038, pp. 97–101, 2011.

[15] X. Qiu, P. Swanson, K. C. Luk et al., "Characterization of antibodies elicited by XMRV infection and development of immunoassays useful for epidemiologic studies," *Retrovirology*, vol. 7, article 68, 2010.

[16] E. Barnes, P. Flanagan, A. Brown et al., "Failure to detect xenotropic murine leukemia virus-related virus in blood of individuals at high risk of blood-borne viral infections," *Journal of Infectious Diseases*, vol. 202, no. 10, pp. 1482–1485, 2010.

[17] S. Tang, J. Zhao, R. Viswanath et al., "Absence of detectable xenotropic murine leukemia virus-related virus in plasma or peripheral blood mononuclear cells of human immunodeficiency virus Type 1-infected blood donors or individuals in Africa," *Transfusion*, vol. 51, no. 3, pp. 463–468, 2011.

[18] N. Onlamoon, J. Das Gupta, P. Sharma et al., "Infection, viral dissemination, and antibody responses of rhesus macaques exposed to the human gammaretrovirus XMRV," *Journal of Virology*, vol. 85, no. 9, pp. 4547–4557, 2011.

[19] S. Palmer, A. P. Wiegand, F. Maldarelli et al., "New real-time reverse transcriptase-initiated PCR assay with single-copy sensitivity for human immunodeficiency virus type 1 RNA in plasma," *Journal of Clinical Microbiology*, vol. 41, no. 10, pp. 4531–4536, 2003.

[20] R. A. Furuta, T. Miyazawa, T. Sugiyama et al., "No association of xenotropic murine leukemia virus-related virus with prostate cancer or chronic fatigue syndrome in Japan," *Retrovirology*, vol. 8, article 20, 2011.

[21] X. Qiu, P. Swanson, N. Tang et al., "Prevalence of XMRV in blood donors, HTLV and HIV cohorts," in *Proceedings of the 15th International Conference on Human Retroviruses: HTLV and Related Viruses. Leuven, Belgium, June, 5-8*, 2011.

[22] G. Simmons, S. A. Glynn, J. A. Holmberg et al., "The Blood Xenotropic Murine Leukemia Virus-Related Virus Scientific Research Working Group: mission, progress, and plans," *Transfusion*, vol. 51, no. 3, pp. 643–653, 2011.

[23] R. S. Arnold, N. V. Makarova, A. O. Osunkoya et al., "XMRV infection in patients with prostate cancer: novel serologic assay and correlation with PCR and FISH," *Urology*, vol. 75, no. 4, pp. 755–761, 2010.

[24] R. Schlaberg, D. J. Choe, K. R. Brown et al., "XMRV is present in malignant prostatic epithelium and is associated with prostate cancer, especially high-grade tumors," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 38, pp. 16351–16356, 2009.

[25] B. P. Danielson, G. E. Ayala, and J. T. Kimata, "Detection of xenotropic murine leukemia virus-related virus in normal and tumor tissue of patients from the southern United States with prostate cancer is dependent on specific polymerase chain reaction conditions," *Journal of Infectious Diseases*, vol. 202, no. 10, pp. 1470–1477, 2010.

[26] K. J. Kunstman, T. Bhattacharya, J. Flaherty et al., "Absence of xenotropic murine leukemia virus-related virus in blood cells of men at risk for and infected with HIV," *AIDS*, vol. 24, no. 11, pp. 1784–1785, 2010.

Hindawi Publishing Corporation
Advances in Virology
Volume 2011, Article ID 797820, 4 pages
doi:10.1155/2011/797820

Research Article

Lack of Detection of Xenotropic Murine Leukemia Virus-Related Virus in HIV-1 Lymphoma Patients

Krista A. Delviks-Frankenberry,[1] Chawaree Chaipan,[1] Rachel Bagni,[2] Kathleen Wyvill,[3] Robert Yarchoan,[3] and Vinay K. Pathak[1]

[1] *Viral Mutation Section, HIV Drug Resistance Program, National Cancer Institute at Frederick, National Institutes of Health, Frederick, MD 21702, USA*
[2] *Protein Expression Laboratory, SAIC-Frederick Inc., NCI-Frederick, Frederick, MD 21702, USA*
[3] *HIV and AIDS Malignancy Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA*

Correspondence should be addressed to Vinay K. Pathak, vinay.pathak@nih.gov

Received 17 June 2011; Accepted 11 July 2011

Academic Editor: Yoshinao Kubo

Xenotropic murine leukemia virus-related virus (XMRV) is a gammaretrovirus reported to be associated with human prostate cancer and chronic fatigue syndrome. Since retroviruses cause various cancers, and XMRV replication might be facilitated by HIV-1 co-infection, we asked whether certain patients with HIV-associated lymphomas are infected with XMRV. Analysis of PMBCs and plasma from 26 patients failed to detect XMRV by PCR, ELISA, or Western blot, suggesting a lack of association between XMRV and AIDS-associated lymphomas.

1. Introduction

A gammaretrovirus, xenotropic murine leukemia virus-related virus (XMRV), was recently discovered and reported to be associated with human prostate cancer (PC) [1]. In the initial report in PC patients, there was a strong correlation between detection of XMRV and a genetic defect in the innate immunity gene *RNASEL* [1]. However, subsequent studies in PC patients showed either no such association [2] or little or no evidence of XMRV infection (reviewed in [3]). Interest in XMRV was increased by the finding that a high percentage of patients with chronic fatigue syndrome (CFS) as well as some asymptomatic patients were infected with XMRV [4]. However, other studies have failed to find such an association (reviewed in [3]). XMRV has been shown to infect peripheral blood mononuclear cells (PBMCs) and CD4+ T cells *in vitro*, indicating that XMRV can infect the same target cells as HIV-1 ([4] and data not shown). However, our recent studies showed that productive replication of XMRV in PBMC and spread in culture are severely restricted by APOBEC3 proteins and perhaps other host defense mechanisms [5]. It remained possible, however, that target cells infected with HIV-1 might provide a favorable environment for XMRV to replicate by depleting cellular host restriction factors such as APOBEC3G, known to inhibit XMRV infection [6, 7]. Several factors led us to explore the possibility that certain patients with HIV-associated lymphoma might be infected with XMRV. Gammaretroviruses can cause lymphomas in other species (reviewed in [8]), and it has been postulated that a number of cases of HIV-associated lymphomas might be caused by an as-yet-unidentified virus [9]. It is also important to note that patients with HIV infection have a higher prevalence of infection with other viruses, and their immunocompromised state might permit more efficient replication of other viruses, including XMRV [9]. Because of the high degree of concern regarding potential XMRV infection and spread in the human population, we sought to investigate whether XMRV might be present in a subset of patients with HIV-associated lymphomas.

Table 1: HIV-1 Lymphoma Patient Cohort Characteristics.

No. of patients	26
Sex, male/female	24/2
Age, median years (range)	38 (21–58)
CD4 count, cells/mm^3, median (range)	76 (0–713)
HIV viremic[a], No. postitive/No. tested	12/21
On anti-HIV drugs	24/26
On AZT	14/26
Lymphomas studied (No. of patients):	PCNSL[b] (11)
	Diffuse large B cell lymphoma (8)
	Burkitts (1)
	Plasmablastic (1)
	Primary effusion lymphoma (1)
	Primary intraocular (1)
	Hodgkin lymphoma (2)
	Head mass- presumptive PCNSL (1)

[a] Detectable p24 Ag or HIV RNA.
[b] Primary Central Nervous System Lymphoma.

2. Analysis and Results

Total nucleic acids from PBMCs were isolated (Qiagen DNA Blood Mini Kit) from 26 HIV-1 infected patients previously diagnosed with AIDS-associated lymphomas (Table 1). The study was approved by the National Cancer Institutional Review Board, and all patients and donors gave written informed consent. Using a real-time quantitative PCR (qPCR) assay that employed primers specific for a unique 24-nucleotide gap in XMRV *gag* (primer and probe sequences as reported in, [10]), 500 ng of patient DNA was tested in each reaction using the Roche LightCycler 480 Probes Master mix. Cycling conditions using the LightCycler 480 Roche instrument (Roche Diagnostics) were 95°C for 30 sec followed by 50 cycles at 95°C for 15 sec and 60°C for 60 sec. Genomic DNA from the XMRV-expressing cell line 22R*v1* (ATCC) was used to generate a standard curve for XMRV (~20 proviral copies/cell). The standard curve was spiked with 500 ng 293T DNA to ensure similar amplification efficiencies as the patient samples. The qPCR had detection sensitivity of <5 XMRV copies/75,757 cells. The quality and quantity of input DNA were confirmed by detection of human GAPDH by qPCR. Using this assay, all 26 HIV-1 lymphoma patients tested were negative for XMRV *gag* sequences (Figure 1).

Patient plasma was also screened by ELISA (Bagni, Protein Expression Laboratory, SAIC-Frederick Inc., NCI-Frederick, unpublished results) for antibodies against XMRV capsid (CA) and envelope (transmembrane, TM) proteins (Figure 2). Due to the absence of definitive XMRV positive patient control samples, sera from macaques before and after

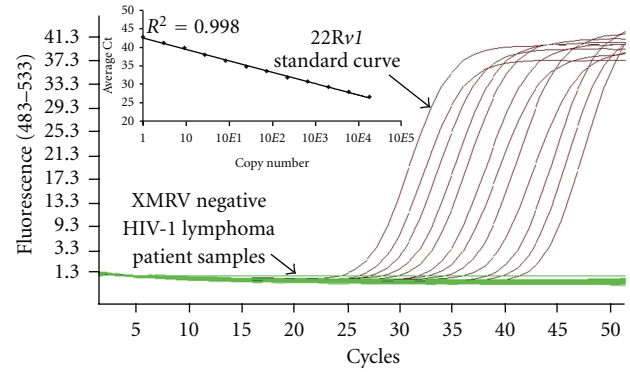


Figure 1: Real-time PCR analysis of HIV-1 lymphoma positive patients for XMRV. XMRV *gag* qPCR failed to detect XMRV from DNA isolated from PBMCs for the 26 HIV-1 lymphoma patients (green lines). Inset shows single-copy sensitivity of the assay.

experimental XMRV infection served as controls for baseline and positive reactivity (Lifson and Del Prete, AIDS and Cancer Virus Program, SAIC-Frederick, Inc., unpublished results). Briefly, plasma samples were collected before and after-inoculation (119 days) from two pigtail macaques inoculated with 4.8×10^9 XMRV RNA equivalents derived from 22R*v1* cell supernatants (Lot SP1592, Biological Products Core, AIDS and Cancer Virus Program, SAIC Frederick, Inc., NCI-Frederick). Samples were considered reactive if they were at least 50% reactive relative to the macaque samples following XMRV infection (positive control sera). None of the 26 HIV-1 lymphoma patient, nor the 10 healthy donors, tested positive for the CA (Figure 2(a)) or TM protein (Figure 2(b)), although 2/26 patients had slight reactivity to TM (asterisk, Figure 2(b)). To evaluate these 2 patients, Western blot analysis was performed to assess whether the TM-reactive ELISA test reflected an immune response to XMRV. Endpoint dilution analysis indicated that a 1 : 2000 dilution of the positive macaque sera produced reproducible positive bands on the western blot with a high signal to noise ratio (Lifson and Del Prete, AIDS and Cancer Virus Program, SAIC-Frederick, Inc., unpublished results). Sera from patients (diluted 1 : 50 or 1 : 200) and positive sera from macaques (diluted 1 : 2000) were tested against XMRV viral lysates obtained from the 22R*v1* cell line. The sera from both TM-reactive patients (10- to 40-fold more concentrated than the macaque) failed to detect capsid, TM, or other XMRV proteins (data not shown), indicating that the TM-positive ELISA test was most likely due to the presence of crossreactive nonspecific antibodies.

3. Discussion

Our studies show a lack of association between XMRV and AIDS-associated lymphomas and complement other studies that have failed to detect XMRV in the PBMCs, plasma, or seminal plasma from HIV-1 infected patients [11–16]. A potential link between XMRV and cancer is hypothesized in that gammaretroviruses can cause sarcomas and leukemias in rodents, felines, and primates (reviewed

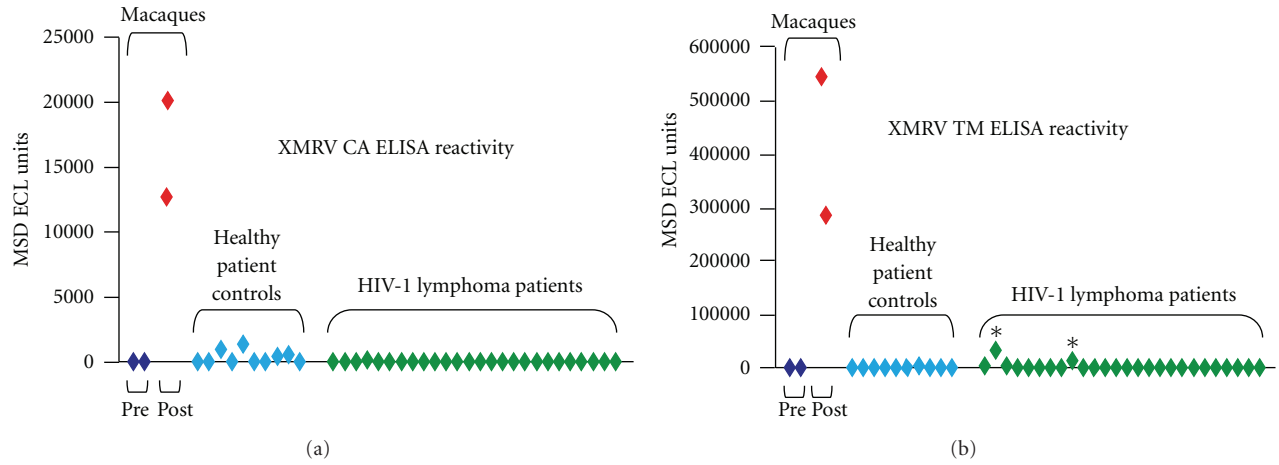


Figure 2: Patient reactivity to XMRV proteins CA and TM. Plasma from the 26 HIV-1 lymphoma positive patients (green diamonds) were assayed by ELISA versus plasma from ten healthy donor controls (light blue diamonds) and two Macaques infected with XMRV pre- (dark blue diamonds) and post- (red diamonds) infection. *indicates two patients with minimal TM reactivity.

in [8]). However, it is unclear whether XMRV infection is associated with prostate or other human cancers. A survey of 800 and 539 PC samples from the United States and Germany, respectively, showed no association with XMRV [17, 18]. A survey of 134 prostate cancer patient plasma samples by us and our collaborators also failed to detect any evidence of XMRV (Kearney et al., under review for this issue of Advances in Virology). Several recent studies have reported that contamination of human samples with mouse DNA may have contributed to XMRV's reported association with human disease [19–22]. Garson et al. [23] also reported that two integration site sequences, previously shown to demonstrate XMRV integration into patient DNA, likely were the result of contamination of identical integration sites from infected DU145 cells reported by the same lab. Furthermore, our recent studies provide compelling evidence that XMRV was generated through recombination of two endogenous murine leukemia viruses during the passage of a PC xenograft in nude mice, and therefore all detection of XMRV from human samples are likely to be due to contamination that originated from this recombination event [24]. It has been hypothesized that this lab-derived virus may have escaped from the lab and is now circulating in the human population. However, our failure to detect XMRV in HIV-1-associated lymphoma patients, along with >10 other studies of different patient cohorts performed by independent investigators, argues against this possibility.

In Summary, we were unable to detect XMRV DNA or XMRV-specific antibodies in the PBMCs or plasma from HIV-1-associated lymphoma patients, further supporting the absence of a link between XMRV and human cancer.

Acknowledgments

The authors thank Wei-Shau Hu for valuable input throughout the project and critical reading of the manuscript and Katie Beam and Allison Meade for technical assistance. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. This work was supported in part by the Intramural Research Program of the National Institutes of Health, National Cancer Institute, Center for Cancer Research, and a Bench-to-Bedside Award to Vinay K. Pathak.

References

[1] A. Urisman, R. J. Molinaro, N. Fischer et al., "Identification of a novel gammaretrovirus in prostate tumors of patients homozygous for R462Q RNASEL variant," *PLoS Pathogens*, vol. 2, no. 3 article e25, pp. 211–225, 2006.

[2] R. Schlaberg, D. J. Choe, K. R. Brown, H. M. Thaker, and I. R. Singh, "XMRV is present in malignant prostatic epithelium and is associated with prostate cancer, especially high-grade tumors," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 38, pp. 16351–16356, 2009.

[3] G. Simmons, S. A. Glynn, J. A. Holmberg et al., "The Blood Xenotropic Murine Leukemia Virus-Related Virus Scientific Research Working Group: mission, progress, and plans," *Transfusion*, vol. 51, no. 3, pp. 643–653, 2011.

[4] V. C. Lombardi, F. W. Ruscetti, J. D. Gupta et al., "Detection of an infectious retrovirus, XMRV, in blood cells of patients with chronic fatigue syndrome," *Science*, vol. 326, no. 5952, pp. 585–589, 2009.

[5] C. Chaipan, K. A. Dilley, T. Paprotka et al., "Severe restriction of xenotropic murine leukemia virus-relate virus replication and spread in cultured human peripheral blood mononuclear cells," *Journal of Virology*, vol. 85, no. 10, pp. 4888–4897, 2011.

[6] T. Paprotka, N. J. Venkatachari, C. Chaipan et al., "Inhibition of xenotropic murine leukemia virus-related virus by APOBEC3 proteins and antiviral drugs," *Journal of Virology*, vol. 84, no. 11, pp. 5719–5729, 2010.

[7] H. C. T. Groom, M. W. Yap, R. P. Galão, S. J. D. Neil, and K. N. Bishop, "Susceptibility of xenotropic murine leukemia virus-related virus (XMRV) to retroviral restriction factors," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 11, pp. 5166–5171, 2010.

[8] N. Maeda, H. Fan, and Y. Yoshikai, "Oncogenesis by retroviruses: old and new paradigms," *Reviews in Medical Virology*, vol. 18, no. 6, pp. 387–405, 2008.

[9] R. Yarchoan, G. Tosato, and R. F. Little, "Therapy insight: AIDS-related malignancies–the influence of antiviral therapy on pathogenesis and management," *Nature Clinical Practice Oncology*, vol. 2, no. 8, pp. 406–415, 2005.

[10] S. Hong, E. A. Klein, J. Das Gupta et al., "Fibrils of prostatic acid phosphatase fragments boost infections with XMRV (Xenotropic Murine leukemia virus-Related Virus), a human retrovirus associated with prostate cancer," *Journal of Virology*, vol. 83, no. 14, pp. 6995–7003, 2009.

[11] K. J. Kunstman, T. Bhattacharya, J. Flaherty, J. P. Phair, and S. M. Wolinsky, "Absence of xenotropic murine leukemia virus-related virus in blood cells of men at risk for and infected with HIV," *AIDS*, vol. 24, no. 11, pp. 1784–1785, 2010.

[12] S. Tang, J. Zhao, R. Viswanath et al., "Absence of detectable xenotropic murine leukemia virus-related virus in plasma or peripheral blood mononuclear cells of human immunodeficiency virus Type 1-infected blood donors or individuals in Africa," *Transfusion*, vol. 51, no. 3, pp. 463–468, 2011.

[13] M. Cornelissen, F. Zorgdrager, P. Blom et al., "Lack of detection of XMRV in seminal plasma from HIV-1 infected men in The Netherlands," *PLoS ONE*, vol. 5, no. 8, Article ID e12040, 2010.

[14] E. Barnes, P. Flanagan, A. Brown et al., "Failure to detect xenotropic murine leukemia virus-related virus in blood of individuals at high risk of blood-borne viral infections," *Journal of Infectious Diseases*, vol. 202, no. 10, pp. 1482–1485, 2010.

[15] T. J. Henrich, J. Z. Li, D. Felsenstein et al., "Xenotropic murine leukemia virus-related virus prevalence in patients with chronic fatigue syndrome or chronic immunomodulatory conditions," *Journal of Infectious Diseases*, vol. 202, no. 10, pp. 1478–1481, 2010.

[16] E. R. Gray, J. A. Garson, J. Breuer et al., "No evidence of XMRV or related retroviruses in a london HIV-1-positive patient cohort," *PLoS ONE*, vol. 6, no. 3, 2011.

[17] A. L. Aloia, K. S. Sfanos, W. B. Isaacs et al., "XMRV: a new virus in prostate cancer?" *Cancer Research*, vol. 70, no. 24, pp. 10028–10033, 2010.

[18] O. Hohn, H. Krause, P. Barbarotto et al., "Lack of evidence for xenotropic murine leukemia virus-related virus(XMRV) in German prostate cancer patients," *Retrovirology*, vol. 6, article no. 1742, p. 92, 2009.

[19] S. Hue, E. R. Gray, A. Gall et al., "Disease-associated XMRV sequences are consistent with laboratory contamination," *Retrovirology*, vol. 7, no. 1 article 111, 2010.

[20] B. Oakes, A. K. Tai, O. Cingoz et al., "Contamination of human DNA samples with mouse DNA can lead to false detection of XMRV-like sequences," *Retrovirology*, vol. 7, article 109, 2010.

[21] M. J. Robinson, O. W. Erlwein, S. Kaye et al., "Mouse DNA contamination in human tissue tested for XMRV," *Retrovirology*, vol. 7, article 108, 2010.

[22] E. Sato, R. A. Furuta, and T. Miyazawa, "An endogenous murine leukemia viral genome contaminant in a commercial RT-PCR Kit is amplified using standard primers for XMRV," *Retrovirology*, vol. 7, article 110, 2010.

[23] J. A. Garson, P. Kellam, and G. J. Towers, "Analysis of XMRV integration sites from human prostate cancer tissues suggests PCR contamination rather than genuine human infection," *Retrovirology*, vol. 8, article 13, 2011.

[24] T. Paprotka, K. A. Delviks-Frankenberry, O. Cingöz et al., "Recombinant Origin of the Retrovirus XMRV," *Science*, vol. 333, no. 6038, pp. 97–101, 2011.

Hindawi Publishing Corporation
Advances in Virology
Volume 2011, Article ID 782353, 6 pages
doi:10.1155/2011/782353

Research Article

No Evidence of XMRV or MuLV Sequences in Prostate Cancer, Diffuse Large B-Cell Lymphoma, or the UK Blood Donor Population

Mark James Robinson,[1] Philip William Tuke,[2] Otto Erlwein,[1] Kate I. Tettmar,[2] Steve Kaye,[1] Kikkeri N. Naresh,[3] Anup Patel,[4] Marjorie M. Walker,[5] Takahiro Kimura,[6] Ganesh Gopalakrishnan,[7] Richard S. Tedder,[2,8] and Myra O. McClure[1]

[1] *Section of Infectious Diseases, Jefferiss Research Trust Laboratories, Imperial College London, St Mary's Campus, London W2 1PG, UK*
[2] *Transfusion Microbiology R&D, National Transfusion Microbiology Laboratories, NHS Blood and Transplant, Colindale, London NW9 5BG, UK*
[3] *Centre for Pathology, Hammersmith Hospital, Imperial College Health Network NHS Trust, 115 1st Floor, L Block, London W12 0HS, UK*
[4] *Urology Department, St Mary's Hospital, Imperial College Healthcare NHS Trust, London W2 1NY, UK*
[5] *Histopathology Department, St Mary's Hospital, Imperial College London, London W2 1NY, UK*
[6] *Department of Urology, The Jikei University School of Medicine, 3-25-8, Nishi-Shinbashi, Minato-ku, Toyko 105-8461, Japan*
[7] *Consultant Urologist, Vedanayagam Hospital, RS Puram, Coimbatore-2 641002, India*
[8] *Blood Borne Viruses Unit, Viralus Reference Department, Centre for Infections, Health Protection Agency, 61 Colindale Avenue, London NW9 5EQ, UK*

Correspondence should be addressed to Mark James Robinson, mark.robinson1@imperial.ac.uk

Received 17 March 2011; Accepted 31 March 2011

Academic Editor: Yoshinao Kubo

Xenotropic murine leukaemia virus-related virus (XMRV) is a recently described retrovirus which has been claimed to infect humans and cause associated pathology. Initially identified in the US in patients with prostate cancer and subsequently in patients with chronic fatigue syndrome, doubt now exists that XMRV is a human pathogen. We studied the prevalence of genetic sequences of XMRV and related MuLV sequences in human prostate cancer, from B cell lymphoma patients and from UK blood donors. Nucleic acid was extracted from fresh prostate tissue biopsies, formalin-fixed paraffin-embedded (FFPE) prostate tissue and FFPE B-cell lymphoma. The presence of XMRV-specific LTR or MuLV generic *gag*-like sequences was investigated by nested PCR. To control for mouse DNA contamination, a PCR that detected intracisternal A-type particle (IAP) sequences was included. In addition, DNA and RNA were extracted from whole blood taken from UK blood donors and screened for XMRV sequences by real-time PCR. XMRV or MuLV-like sequences were not amplified from tissue samples. Occasionally MuLV *gag* and XMRV-LTR sequences were amplified from Indian prostate cancer samples, but were always detected in conjunction with contaminating murine genomic DNA. We found no evidence of XMRV or MuLV infection in the UK blood donors.

1. Introduction

In 2006, a new gammaretrovirus, xenotropic murine leukaemia virus-related virus (XMRV), was discovered by the Virochip analysis in prostate cancer tissue from patients homozygous for an RNase L mutation [1]. In these patients, the innate antiviral defence RNase L pathway is defective; hence, these patients are likely to be susceptible to viral infection and a population more likely to find a novel virus with disease association in. When a second US study found that 6% of all prostate cancer patients, independent of RNase L mutations, were infected with the virus, thus broadening the population at risk [2], interest in XMRV intensified. However, subsequent studies from the USA [3, 4] and all

European studies [5–7] failed to confirm the presence of XMRV in prostate tissue. More recently it has been suggested that XMRV detection in prostate tissue in the US could be related to the specificity and conditions of the PCR used [8].

In 2009, Lombardi and colleagues reported the presence of XMRV proviral DNA in peripheral blood leucocytes from 3.7% of healthy controls and 67% of patients with chronic fatigue syndrome (CFS) [9]. The detection rate by PCR amplification of XMRV proviral DNA subsequently reduced the estimated CFS prevalence to 7%, with the explanation that RNA extraction and cDNA synthesis had been required to achieve the 67% prevalence originally reported [10]. Lo and colleagues (2010) using predominantly archival material from patients with CFS detected a high prevalence (86.5%) of pMuLVs. These are similar to, but constitute a different group from, the xenotropic endogenous MuLVs to which XMRV belongs [11]. However, questions were raised about how these data were generated [12], and a number of other studies have failed to demonstrate a link between XMRV or pMuLV infection and CFS [13–19].

The causes of B-cell lymphoma are not fully understood [20], but the clinical and epidemiological characteristics are suggestive of the involvement of an infectious agent [21]. Several viruses [22, 23] have been linked to the risk of B-cell lymphoma, most notably EBV [24–26], and retroviruses are implicated in animal leukaemias. Retroviral integration could cause somatic DNA changes leading to clonal expansion of B cells resulting in leukaemia as has been previously described for adult T-cell leukaemia (ATL) and HTLV-1 [27].

The geographical discrepancy of XMRV and pMuLV prevalence remains unexplained. To explore this further, we have tested a variety of tissues from diverse populations; prostate cancer (PC) formalin-fixed paraffin-embedded (FFPE) tissue from Japan and India, fresh prostate tissue samples received from the Urology Clinic at St Mary's Hospital, London, and peripheral blood from English blood donors.

A series of recent papers [28–31] have demonstrated the ease with which specimens can be contaminated with murine DNA sequences. To control for this, all tissue specimens were tested by PCR specific for intracisternal A particle (IAP), a retrotransposon present in multiple copies (~1000) within the mouse genome [32].

2. Methods and Materials

2.1. Samples and Nucleic Acid Isolation. Prostate biopsies were collected from 55 patients admitted to the Urology Department, St. Mary's Hospital, London, UK to undergo routine biopsy for prostate cancer screening. All patients gave written informed consent for their tissue to be banked for the purposes of research (ethics number 99/CCC/166, August 1999). The DNA was extracted using the QIAamp DNA mini kit (Qiagen, Crawley, UK) following the manufacturer's instructions.

B-cell lymphoma samples were provided by Professor Kikkiri Naresh, Centre for Pathology, Hammersmith Hospital, London, UK. The DNA from 10 Diffuse Large B-cell Lymphoma (DLBLC) patients was extracted from FFPE tissues of lymph nodal or extranodal diffuse large B-cell lymphoma using the DNeasy Blood & Tissue Kit (Qiagen). Briefly, two 15 μm sections were cut and transferred to 1.5 mL Eppendorf tubes. Blades were changed between samples to avoid cross-contamination. Sections were deparaffinised with xylene and ethanol, rehydrated, and incubated with proteinase K and lysis buffer in a shaking water bath at 55°C overnight and the extraction was completed according to the manufacturer's instructions.

Twenty FFPE prostate specimens including 10 prostate cancer (PC) and 10 benign prostatic hyperplasia (BPH) samples were supplied by Professor Ganesh Golpalakrishnan of Vedanayagam Hospital, RS Puram, Coimbatore, India and sixteen specimens from Dr. Takahiro Kimura of the Department of Urology, The Jikei University School of Medicine, Japan. From the Indian blocks, two 10 μM sections were extracted with the QIAamp DNA FFPE tissue kit (Qiagen), according to the manufacturer's instructions. The Japanese samples were provided presliced on glass slides.

Random anonymous whole-blood samples were obtained from the Donation Testing Department at the National Health Service Blood and Transplant (NHSBT) Centre at Colindale, London, UK. Plasma minipools were similarly obtained from NHSBT. All blood and plasma samples were extracted on a Qiagen MDx Biorobot and eluted with 80 μL of Qiagen buffer AVE.

2.2. XMRV, MuLV, and Control Nested PCR. Samples were tested for the presence of XMRV and MuLV proviral DNA using nested PCR, as described previously [14]. Briefly, we used a set of primers that encompasses the 24 bp deletion in the XMRV *gag* leader region, originally described to distinguish XMRV as a new human virus, along with a second set of primers reflecting a sequence conserved amongst most MuLVs. The positive control for the XMRV and MuLV PCRs was plasmid VP62 [1]. The PCR method has been shown to be sensitive enough to pick up one copy of XMRV VP62 plasmid in a background of 500 ng DNA [28]. As a control for sample addition and PCR inhibition, primers to the human beta-globin (hBG) gene were used. DNA extracted from LNCaP (human prostate cancer cells) was used as a positive control for human beta globin. To control for contamination of samples with murine DNA, primers specific to mouse IAP were used as described previously [28]. The positive control for IAP was DNA from the McCoy cell (murine fibroblast cells, ECAAC 90010305). In all PCRs, at least 6 "no template" controls were set up. All PCR products were visualised on Ethidium Bromide-stained 2% agarose gels.

2.3. XMRV, MuLV, and Control Real-Time PCRs for Blood Donor Studies. Real-time PCR was performed as detailed in Table 1. For the proviral DNA analysis, 10 μL of the nucleic acid extract were analysed separately in three individual quantitative PCRs (Q-PCRs).

2.3.1. XMRV Q-PCR and Internal Control. Samples were tested by Q-PCR for XMRV, as described by McCormick et al. [33] and modified as detailed in Table 1. In a Q-PCR to

Table 1: Details of PCRs used to test blood sample.

Sample tested	PCR	Target	Primers and probes	Cycles (N)	Reagents
540 DNAs from whole blood	XMRV Taq Man	gag	XMRV Probe, F, R	60	Qiagen QuantiTect Probe kit
540 DNAs from whole blood	SBCMV Taq Man	SBCMV plasmid	SBCWMVCPF, SBCWMVCPR, SBWMV237F	45	ABgene ABsolute QPCR ROX mastermix
540 DNAs from whole blood	PDH Taq Man	PDH human gene	PDH Probe, F, R	45	ABgene ABsolute QPCR ROX mastermix
600 NAs from whole blood 400 NAs from plasma minipools	XMRV/pMuLV RT Taq Man with BMV RT Taq Man	gag BMV	P2, F3, R4 BMV Probe, F, R	45	Qiagen QuantiTect Probe RT-PCR kit

The TaqMan assay conditions were 15 min at 95°C (15 secs 95°C, 1 min 60°C) $\times N$ cycles. 400 nM concentrations of primers, and 200 nM probes were used in all the TaqMan assays with the exception of the CDC MuLV Taq Man where the concentration of each probe was 100 nM and the PDH TaqMan where the primer concentrations were 50 nM.

control for the extraction efficiency and amplification inhibition coextracted soil-borne cereal mosaic virus (SBCMV) plasmid DNA was used, (5.4×10^6 copies were added to the 33 mL of Qiagen lysis buffer AL used for extracting 96 samples on the MDx Biorobot). This reaction was as described by Ratti et al. [34]. The primer sequences for this reaction were *SBCWMVCPF* (5′-CAC TCA GGA CGG TGA CGA GAT-3′), SBCWMVCPR (5′-GTG ATA CTG TGA GTC TGG TGA TGA TTT-3′) and probe *SBWMV237Fa* (5′ JOE-TTT TGT GAC CTT GGA GGT GAG GCA GTT ATG-BHQ1-3′).

2.3.2. Q-PCR for Quantification of Human DNA. The input of human DNA in each extract was measured by a Q-PCR for the Pyruvate dehydrogenase (PDH) gene. Primers used *PDH Taq 1* (5′-TGA AAG TTA TAC AAA ATT GAG GTC ACT GTT-3′), *PDH Taq 2* (5′- TCC ACA GCC CTC GAC TAA CC -3′) with probe *PDHP* (5′-VIC-CCC CCA GAT ACA CTT AAG GGA TCA ACT CTT AAT TGT-Tamra-3′). Positive control for this reaction was a dilution series of human male DNA (Applied Biosystems, Warrington, UK, Catalogue no. 4312660). The XMRV Q-PCR results were validated when the PDH threshold cycle (Ct) value was greater than the mean Ct minus 3 SD, and the SBCMV control was greater than the mean Ct minus 2 SD. Samples invalid on either control were excluded from the analysis.

2.4. Detection of Gag Sequences by Nested PCR in Blood Donors. Nuclease-free water (Severn Biotech, Kidderminster, UK) was used throughout for the cDNA and PCR mix preparations and as no-template controls. Nucleic acid extracts were tested by nested PCR using the *gag* primers as described by Lombardi et al. [9] and Lo et al. [11], but using Applied Biosystems Taq Gold LD PCR enzyme (Table 1) to overcome the problem of false positives that have arisen from the use of Invitrogen Taq Polymerase [30].

2.5. QRT-PCR Amplification of XMRV/pMuLV in Blood Donors. An XMRV/pMuLV *gag* QRT-PCR assay described by Lo and colleagues [11] but modified to detect the pMuLVs was used to test nucleic acid from whole blood, plasma, and from plasma minipools. Further details of all QPCR and QRT-PCR reactions are listed in Table 1. The primers for this reaction were *F3* (5′-ACC GTT TGT CTC TCC TAA AC-3′) and *R4* (5′-AGG GTA AAG GGC AGA TCG-3′), with probe *P2* (5′-Fam-CCG ACA GCT CCC GTC CTC CCG-Tamra-3′). Nuclease-free water (Severn Biotech) was used throughout for the RT-PCR mix preparations and as no template controls. RT-PCR was performed in a total volume of 50 μL, containing 1x Qiagen QuantiTect RT-PCR buffer and primers, and probes as detailed in Table 1. Synthesis conditions were 50°C for 30 mins, followed by 95°C for 15 mins and 45 cycles of 95°C for 15 secs 60°C for 1 min. Twenty μl of nucleic acid was analysed in a QRT-PCR which multiplexed the XMRV/pMuLV TaqMan with the internal control TaqMan reaction (Brome mosaic virus (BMV)) [35]. The BMV RNA was added to the Qiagen AL lysis buffer and co-extracted with the sample. A sample was valid if the BMV Ct value was greater than the mean Ct minus 2 SD. Samples invalid on the BMV control were excluded from the analysis. The sensitivity of this QRT-PCR was determined as 150 RNA copies/mL (75 viral particles/mL) by calculation from the observed frequency of negatives using the Poisson distribution.

3. Results

3.1. XMRV Detection in Tissue Samples by Nested PCR. A representative stained gel following nested PCR is shown in Figure 1. For routine analysis, 0.11 pg of plasmid DNA (representing approximately 7000 copies/PCR) was used as positive control for XMRV and MuLV. All samples were positive for hBG sequences by PCR. The sensitivity of the IAP PCR has been shown previously to detect as little as 0.0011 pg DNA in a background of 500 ng DNA [28]. The results are summarised in Table 2(a). No evidence of XMRV or MuLV was found in any of the FFPE prostate tissue samples from Japan or the fresh prostate tissues from the

Table 2

(a) Amplification from fresh and FFPE tissues by nested PCR

	Fresh prostate tissue	Japan samples	Indian samples	LCBCL samples
Number of cancerous samples	16/55	16	10/20	10
Number of noncancerous samples (unknown status)	18/55 (21/55)	0	10/20	0
Mean age (range)	unknown	unknown	72 (62–85)	43 (27–83)
Beta globin +	55/55	16/16	20/20	10/10
XMRV +	0	0	2/20	0
MuLV +	0	0	4/20	0
IAP +	0	0	5/20	0
mtDNA +	nd	nd	2/10	nd

(b) Specific PCR results from Indian samples

Indian sample number	Cancer status	PCR result using specific primers				
		β-globin	IAP	mtDNA	MLV *gag*	XMRV LTR
6489c/10	cancer	+	+	+	+	+
5383c/10	cancer	+	+	−	+	+
5406a3/10	cancer	+	+	−	−	−
2896c/10	BPH	+	+	+	+	−
5349c/10	cancer	+	+	−	+	−

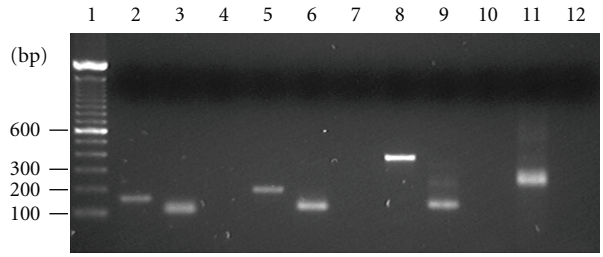


Figure 1: lane 1: MWM; lanes 2–4: β-globin PCR on LNCaP DNA template 1st round product, 2nd round product, and no-template control; lanes 5–7: XMRV LTR PCR on VP62 plasmid DNA template 1st round product, 2nd round product, and no-template control; lanes 8–10: MuLV *gag* PCR on VP62 plasmid DNA template 1st round product, 2nd round product, and no-template control; lanes 11-12: IAP PCR on McCoy cell DNA template and no-template control.

UK. Of the 20 Indian samples, four (20%) produced a PCR signal with the MuLV *gag* primers (three prostate cancer, one benign prostatic hyperplasmia) and of these, 2/4 were positive with XMRV LTR primers (both prostate cancer). The IAP PCR was applied to the same samples to see if the positive signal was due to mouse DNA contamination. All MuLV/XMRV amplification was concordant with IAP amplification, except for one prostate cancer sample which was positive for IAP without MuLV/XMRV amplification. Confirmation of murine DNA contamination was achieved using PCR primers specific to mouse mitochondrial DNA (mtDNA). Although this PCR has been shown to be less sensitive than IAP PCR [28], 2/20 of the Indian samples (one prostate cancer, one benign prostatic hyperplasia) were positive for mtDNA. In both of these samples, IAP and MuLV *gag* sequences were amplified. Additionally, one was positive for XMRV (detailed in Table 2(b)).

No evidence of MuLV or XMRV sequences was discovered in the DLBCL samples and none of the DCBCL samples gave an IAP specific product.

3.2. XMRV Detection in Whole Blood by Real-Time PCR. XMRV proviral DNA was not amplified from whole-blood extracts derived from 540 donors. The average DNA input for each amplification was 93,000 cells (approx 0.56 μg). Detection of XMRV/MuLV RNA was undertaken on a further 600 donors and 400 plasma minipools, derived from 19,200 individual donations. All samples tested negative for XMRV and MuLV sequences.

4. Discussion

Using highly sensitive PCRs with primers that detect XMRV and primers that detect MuLV-like sequences, no proviral DNA was detected in any of the prostate cancer samples independently of murine DNA contamination. This served to confirm our previous studies in which FFPE prostate tissue was tested and XMRV/MuLV sequences failed to be amplified [28]. Here we have added further data to show that no XMRV or MuLV-like sequences can be detected in fresh UK prostate tissue or in prostate cancer samples collected from Japan. Samples from India showed evidence of MuLV and XMRV sequences when viral genomic sequences were amplified by nested PCR. However, this was concordant with murine genomic DNA contamination detected using primers

to IAP. IAPs are retrotransposons present at the level of around 1000 copies per mouse genome [30]. Thus, IAP PCR represents a highly sensitive detection method for murine DNA. Although the sample size was small ($n = 10$), we found no evidence to suggest that XMRV might be involved in other cancers, such as diffuse large B-cell lymphoma.

It was reported last year that XMRV had been detected in greater than 60% of 50 samples from English blood donors [36]. In contrast, we found no evidence of XMRV or pMuLV in any of 540 whole-blood samples from unselected NHSBT donors nor were we able to detect MuLV-like sequences in either the DNA from whole blood or cDNA prepared from the plasma minipools from donors in England. There are three possible explanations for this. Firstly, there are no MuLV infections in blood donors in England. Secondly, there are MuLV infections, but that the assays used failed to detect them, either due to sensitivity or sequence variation. Thirdly, there are MuLV infections, but the prevalence is too low to be detected in the sample sizes tested.

Research into the presence of MuLVs in the human population is contentious, given discrepant findings [37–39]. Contamination from sequences contained in apparently XMRV-positive samples, amplified products, or plasmids has been suggested as a reason for the finding of MuLVs in human samples [30, 40]. A study of XMRV in patients with CFS or chronic immunomodulatory conditions, using Invitrogen Platinum Taq (IPT), reported a *gag* sequence with >99% homology to a mouse endogenous retrovirus [19]. This was designated as contamination, although the paper failed to speculate on the source of this sequence. Sato and colleagues (2010) recently reported finding predominantly RNA sequences, related to a pMuLV, in IPT containing reagents [30]. Another study concluded that the detection of MuLV-related sequences in human samples could be due to contamination with mouse DNA, most likely contained in various laboratory reagents [29]. We have demonstrated that murine sequences can be present in prostate sections, resulting in false positive detection of XMRV [28]. A phylogenetic overview concluded that the proviral sequences present in the genome of 22Rv1 cell line were ancestral to the published XMRV sequences [31]; finally, it has been shown that the mapping of integration sites of XMRV in prostate cancer tissues, thought to unequivocally confirm the existence of XMRV in clinical samples, was at least partially contaminant derived [41], further emphasising the ease with which contamination can occur.

The sources of contamination are still to be fully elucidated. However, given that most retroviral laboratories have worked with MuLV or MuLV-derived vector systems, or at least used murine reagents, it is essential that sufficient appropriate controls are included in all PCRs.

The absence of MuLVs from all the samples analysed in this study, where there was no concomitant detection of murine genomic sequences, adds weight to the growing body of data questioning the evidence for murine retrovirus infection of humans [42]. It is always challenging to prove a negative result, but it is likely that XMRV will be added to the long list of RNA rumour viruses [43].

Conflict of Interests

The authors declare no conflict of interests.

Acknowledgments

The authors would like to thank Dr. R. Silverman for the generous donation of the VP62 plasmid and virus used as positive controls for our PCR and RT-PCR assays. They would like to thank Dr. PG Grant for the design of the PDH TaqMan assay. They would like to thank Dr. Giles Budge for providing the plasmid containing the cloned SBCMV PCR product. They would like to thank Ms Poorvi Patel for assisting with the QRT-PCRs and Ms Renata Szypulska for assisting with the robotic extractions and Miss Mahrokh Nohadan for preparing the FFPE tissue slices. They are grateful for support from the NIHR Biomedical Research Centre funding scheme and the NHS Blood and Transplant for funding this research. M. J. Robinson and P. W. Tuke contributed equally to this study.

References

[1] A. Urisman, R. J. Molinaro, N. Fischer et al., "Identification of a novel gammaretrovirus in prostate tumors of patients homozygous for R462Q RNASEL variant," *PLoS Pathogens*, vol. 2, no. 3, pp. 211–225, 2006.

[2] R. Schlaberg, D. J. Choe, K. R. Brown, H. M. Thaker, and I. R. Singh, "XMRV is present in malignant prostatic epithelium and is associated with prostate cancer, especially high-grade tumors," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 38, pp. 16351–16356, 2009.

[3] A. L. Aloia, K. S. Sfanos, W. B. Isaacs et al., "XMRV: a new virus in prostate cancer?" *Cancer Research*, vol. 70, no. 24, pp. 10028–10033, 2010.

[4] S. Sabunciyan, N. Mandelberg, C. S. Rabkin, R. Yolken, and R. Viscidi, "No difference in antibody titers against xenotropic MLV related virus in prostate cancer cases and cancer-free controls," *Molecular and Cellular Probes*, vol. 25, no. 2-3, pp. 134–136, 2011.

[5] N. Fischer, O. Hellwinkel, C. Schulz et al., "Prevalence of human gammaretrovirus XMRV in sporadic prostate cancer," *Journal of Clinical Virology*, vol. 43, no. 3, pp. 277–283, 2008.

[6] F. R. D'Arcy, A. Foley, L. Perry et al., "No evidence of XMRV in Irish prostate cancer patients with the R462Q mutations," *European Urology*, vol. 7, supplement 271, 2008.

[7] O. Hohn, H. Krause, P. Barbarotto et al., "Lack of evidence for xenotropic murine leukemia virus-related virus(XMRV) in German prostate cancer patients," *Retrovirology*, vol. 6, article 92, 2009.

[8] B. P. Danielson, G. E. Ayala, and J. T. Kimata, "Detection of xenotropic murine leukemia virus-related virus in normal and tumor tissue of patients from the southern United States with prostate cancer is dependent on specific polymerase chain reaction conditions," *Journal of Infectious Diseases*, vol. 202, no. 10, pp. 1470–1477, 2010.

[9] V. C. Lombardi, F. W. Ruscetti, J. D. Gupta et al., "Detection of an infectious retrovirus, XMRV, in blood cells of patients with chronic fatigue syndrome," *Science*, vol. 326, no. 5952, pp. 585–589, 2009.

[10] J. A. Mikovits, V. C. Lombardi, M. A. Pfost, K. S. Hagen, and F. W. Ruscetti, "Detection of an infectious retrovirus, XMRV,

in blood cells of patients with chronic fatigue syndrome," *Virulence*, vol. 1, no. 5, pp. 386–390, 2010.

[11] S.-C. Lo, N. Pripuzova, B. Li et al., "Detection of MLV-related virus gene sequences in blood of patients with chronic fatigue syndrome and healthy blood donors," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 36, pp. 15874–15879, 2010.

[12] O. Erlwein, S. Kaye, M. Robinson, and M. McClure, "Chronic fatigue syndrome: xenotropic murine leukemia virus-related virus, murine leukemia virus, both, or neither?" *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 43, p. E161, 2010.

[13] W. M. Switzer, H. Jia, O. Hohn et al., "Absence of evidence of Xenotropic Murine Leukemia Virus-related virus infection in persons with Chronic Fatigue Syndrome and healthy controls in the United States," *Retrovirology*, p. 57, article 57, 2010.

[14] O. Erlwein, S. Kaye, M. O. McClure et al., "Failure to detect the novel retrovirus XMRV in chronic fatigue syndrome," *PLoS One*, vol. 5, no. 1, Article ID e8519, 2010.

[15] H. C. T. Groom, V. C. Boucherit, K. Makinson et al., "Absence of xenotropic murine leukaemia virus-related virus in UK patients with chronic fatigue syndrome," *Retrovirology*, vol. 7, article no. 10, 2010.

[16] P. Hong, J. Li, and Y. Li, "Failure to detect Xenotropic murine leukaemia virus-related virus in Chinese patients with chronic fatigue syndrome," *Virology Journal*, vol. 7, article 224, 2010.

[17] F. J. van Kuppeveld, A. S. de Jong, K. H. Lanke et al., "Prevalence of xenotropic murine leukaemia virus-related virus in patients with chronic fatigue syndrome in the Netherlands: retrospective analysis of samples from an established cohort," *BMJ*, vol. 340, p. c1018, 2010.

[18] W. M. Switzer, H. Jia, O. Hohn et al., "Absence of evidence of Xenotropic Murine Leukemia Virus-related virus infection in persons with Chronic Fatigue Syndrome and healthy controls in the United States," *Retrovirology*, vol. 7, article 57, 2010.

[19] T. J. Henrich, J. Z. Li, D. Felsenstein et al., "Xenotropic murine leukemia virus-related virus prevalence in patients with chronic fatigue syndrome or chronic immunomodulatory conditions," *Journal of Infectious Diseases*, vol. 202, no. 10, pp. 1478–1481, 2010.

[20] C. R. Flowers, R. Sinha, and J. M. Vose, "Improving outcomes for patients with diffuse large B-cell lymphoma," *CA Cancer Journal for Clinicians*, vol. 60, no. 6, pp. 393–408, 2010.

[21] S. G. Fisher and R. I. Fisher, "The epidemiology of non-Hodgkin's lymphoma," *Oncogene*, vol. 23, no. 38, pp. 6524–6534, 2004.

[22] Y. Allory, D. Challine, C. Haioun et al., "Bone marrow involvement in lymphomas with hemophagocytic syndrome at presentation: a clinicopathologic study of 11 patients in a Western institution," *American Journal of Surgical Pathology*, vol. 25, no. 7, pp. 865–874, 2001.

[23] A. Carbone, E. Cesarman, M. Spina, A. Gloghini, and T. F. Schulz, "HIV-associated lymphomas and gamma-herpesviruses," *Blood*, vol. 113, no. 6, pp. 1213–1224, 2009.

[24] H. H. Wong and J. Wang, "Epstein-Barr virus positive diffuse large B-cell lymphoma of the elderly," *Leukemia and Lymphoma*, vol. 50, no. 3, pp. 335–340, 2009.

[25] A. Carbone, A. Gloghini, and G. Dotti, "EBV-associated lymphoproliferative disorders: classification and treatment," *Oncologist*, vol. 13, no. 5, pp. 577–585, 2008.

[26] D. A. Thorley-Lawson and A. Gross, "Persistence of the Epstien-Barr Virus and the origins of associated lymphomas," *New England Journal of Medicine*, vol. 350, no. 13, pp. 1328–1337, 2004.

[27] G. P. Taylor and M. Matsuoka, "Natural history of adult T-cell leukemia/lymphoma and approaches to therapy," *Oncogene*, vol. 24, no. 39, pp. 6047–6057, 2005.

[28] M. J. Robinson, O. W. Erlwein, S. Kaye et al., "Mouse DNA contamination in human tissue tested for XMRV," *Retrovirology*, vol. 7, article 108, 2010.

[29] B. Oakes, A. K. Tai, O. Cingöz et al., "Contamination of human DNA samples with mouse DNA can lead to false detection of XMRV-like sequences," *Retrovirology*, vol. 7, article 109, 2010.

[30] E. Sato, R. A. Furuta, and T. Miyazawa, "An endogenous murine leukemia viral genome contaminant in a commercial RT-PCR kit is amplified using standard primers for XMRV," *Retrovirology*, vol. 7, article 110, 2010.

[31] S. Hué, E. R. Gray, A. Gall et al., "Disease-associated XMRV sequences are consistent with laboratory contamination," *Retrovirology*, vol. 7, article 111, 2010.

[32] A. Dupressoir and T. Heidmann, "Expression of intracisternal A-particle retrotransposons in primary tumors of oncogene-expressing transgenic mice," *Oncogene*, vol. 14, no. 24, pp. 2951–2958, 1997.

[33] A. L. McCormick, R. H. Brown, M. E. Cudkowicz, A. Al-Chalabi, and J. A. Garson, "Quantification of reverse transcriptase in ALS and elimination of a novel retroviral candidate," *Neurology*, vol. 70, no. 4, pp. 278–283, 2008.

[34] C. Ratti, G. Budge, L. Ward, G. Clover, C. Rubies-Autonell, and C. Henry, "Detection and relative quantitation of Soil-borne cereal mosaic virus (SBCMV) and Polymyxa graminis in winter wheat using real-time PCR (TaqMan®)," *Journal of Virological Methods*, vol. 122, no. 1, pp. 95–103, 2004.

[35] R. B. Ferns and J. A. Garson, "Development and evaluation of a real-time RT-PCR assay for quantification of cell-free human immunodeficiency virus type 2 using a Brome Mosaic Virus internal control," *Journal of Virological Methods*, vol. 135, no. 1, pp. 102–108, 2006.

[36] J. P. Stoye, R. H. Silverman, C. A. Boucher, and S. F. J. Le Grice, "The xenotropic murine leukemia virus-related retrovirus debate continues at first international workshop," *Retrovirology*, vol. 7, article 113, 2010.

[37] R. A. Smith, "Contamination of clinical specimens with MLV-encoding nucleic acids: implications for XMRV and other candidate human retroviruses," *Retrovirology*, vol. 7, article 112, 2010.

[38] Editorial, "Cause for concern," *Nature*, vol. 471, p. 366, 2011.

[39] L. Menéndez-Arias, "Evidence and controversies on the role of XMRV in prostate cancer and chronic fatigue syndrome," *Reviews in Medical Virology*, vol. 21, no. 1, pp. 3–17, 2011.

[40] P. W. Tuke, K. I. Tettmar, A. Tamuri, J. P. Stoye, and R. S. Tedder, "PCR Master Mixes Harbour Murine DNA Sequences," *Caveat Emptor! PLoS ONE*, vol. 6, no. 5, p. e19953, 2011.

[41] J. A. Garson, P. Kellam, and G. J. Towers, "Analysis of XMRV integration sites from human prostate cancer tissues suggests PCR contamination rather than genuine human infection," *Retrovirology*, vol. 8, article 13, 2011.

[42] J. Cohen, "Retrovirology: more negative data for link between mouse virus and human disease," *Science*, vol. 331, no. 6022, pp. 1253–1254, 2011.

[43] R. A. Weiss, "A cautionary tale of virus and disease," *BMC Biology*, vol. 8, article 124, 2010.

Hindawi Publishing Corporation
Advances in Virology
Volume 2011, Article ID 272193, 12 pages
doi:10.1155/2011/272193

Research Article

Nucleic Acid, Antibody, and Virus Culture Methods to Detect Xenotropic MLV-Related Virus in Human Blood Samples

M. F. Kearney,[1] K. Lee,[1] R. K. Bagni,[2] A. Wiegand,[1] J. Spindler,[1] F. Maldarelli,[1] P. A. Pinto,[3] W. M. Linehan,[3] C. D. Vocke,[3] K. A. Delviks-Frankenberry,[1] R. W. deVere White,[4] G. Q. Del Prete,[5] J. W. Mellors,[6] J. D. Lifson,[5] V. N. KewalRamani,[1] V. K. Pathak,[1] J. M. Coffin,[7] and S. F. J. Le Grice[1]

[1] *HIV Drug Resistance Program, National Cancer Institute at Frederick, Frederick, MD 21702-1201, USA*
[2] *Protein Expression Laboratory, SAIC-Frederick, Inc., NCI-Frederick, Frederick, MD 21702, USA*
[3] *Urologic Oncology Branch, National Cancer Institute, Bethesda, MD 20892, USA*
[4] *UC Davis Cancer Center, Sacramento, CA 95817, USA*
[5] *AIDS and Cancer Virus Program, SAIC-Frederick, Inc., National Cancer Institute, Frederick, MD 21702, USA*
[6] *Department of Medicine, University of Pittsburgh, Pittsburgh, PA 15260, USA*
[7] *Department of Molecular Biology and Microbiology, Tufts University, Boston, MA 02155, USA*

Correspondence should be addressed to M. F. Kearney, kearneym@mail.nih.gov

Received 21 June 2011; Revised 8 August 2011; Accepted 27 August 2011

Academic Editor: Yoshinao Kubo

The MLV-related retrovirus, XMRV, was recently identified and reported to be associated with both prostate cancer and chronic fatigue syndrome. At the National Cancer Institute-Frederick, MD (NCI-Frederick), we developed highly sensitive methods to detect XMRV nucleic acids, antibodies, and replication competent virus. Analysis of XMRV-spiked samples and/or specimens from two pigtail macaques experimentally inoculated with 22Rv1 cell-derived XMRV confirmed the ability of the assays used to detect XMRV RNA and DNA, and culture isolatable virus when present, along with XMRV reactive antibody responses. Using these assays, we did not detect evidence of XMRV in blood samples ($N = 134$) or prostate specimens ($N = 19$) from two independent cohorts of patients with prostate cancer. Previous studies detected XMRV in prostate tissues. In the present study, we primarily investigated the levels of XMRV in blood plasma samples collected from patients with prostate cancer. These results demonstrate that while XMRV-related assays developed at the NCI-Frederick can readily measure XMRV nucleic acids, antibodies, and replication competent virus, no evidence of XMRV was found in the blood of patients with prostate cancer.

1. Introduction

Xenotropic murine leukemia virus-related virus (XMRV) is a recently discovered gammaretrovirus reportedly associated with prostate cancer and chronic fatigue syndrome (CFS) [1, 2]. The discovery of XMRV arose from studies investigating a potential viral cause for diseases in patients with an *RNAseL* gene variant. This genotype, which is observed in a varying subset of patients in cohorts with prostate cancer [1, 3–8], has been associated with impairment of innate immune responses to viral infections [5]. Seeking an etiologically significant viral infection associated with impaired *RNAse L*-dependent responses, Urisman et al. first identified XMRV in 2006 in a cohort of prostate cancer patients [2]. The association of XMRV with prostate cancer, but not its association with the *RNAseL* variant, was corroborated by Schlaberg et al. in 2009 [9]. The prostate cancer studies were followed by a report from Lombardi et al. presenting evidence for XMRV infection in 67% of individuals with severe CFS, compared to 3.7% of healthy individuals [1]. These high reported frequencies of XMRV infection and putative linkage to a debilitating illness prompted concerns about the possibility of a new, widespread retroviral epidemic and stimulated additional research towards determining the prevalence of XMRV infection in different populations worldwide.

Several studies supporting high prevalence of XMRV infection followed. For example, Arnold et al. detected anti-XMRV antibodies in 27% of individuals with prostate cancer [10], Schlaberg et al. found XMRV nucleic acid in 23% of prostate cancers and 4% of controls [11], and Danielson et al. detected XMRV in 22.8% of extracted prostate tissues from individuals who had radical prostatectomies [12]. However, controversy arose when other laboratories could not demonstrate comparable findings in similar cohorts not only in the US [13] but in Germany [14], The Netherlands [15], and England [16, 17]. Adding to the controversy, Lo et al. reported the presence of mouse retroviral sequences, but not XMRV, in 86.5% of CFS patients [18]. Claims were made that such findings supported the association of XMRV infection with CFS, complicating an already controversial field.

Several factors were speculatively proposed to contribute to the differential detection of XMRV/MLVs by different laboratories. It was suggested that inconsistencies in detection of XMRV/MLVs in patient samples could result from varied prevalence of infection in different populations, differing criteria for patient selection, and differing detection methodologies utilized [19]. It was also proposed that virus levels may be chronically low or episodic in patient plasma or tissues, making virus detection difficult [19]. Adding to the complexity, detection of XMRV by PCR is highly susceptible to false positive results due to the very close genetic relationship of XMRV with endogenous MLVs and the high prevalence of contaminating mouse genomic DNA in many specimens [20, 21]. Indeed, studies have suggested that XMRV detection is the result of laboratory contamination from infected cell lines [22–25] or contaminated reagents [26]. Further suggestions of laboratory contamination came after publication of a study by Paprotka et al. [25], showing that XMRV originated in a human cancer cell line generated by passaging prostate cancer cells through immunocompromised mice. This result indicates that XMRV could not have entered the human population until recently, yet was already being reported as prevalent in a sizeable fraction of prostatic cancers. Furthermore, it showed that most "XMRV-specific" detection assays could, in fact, detect one or the other of the two parental proviruses (PreXMRV-1 and 2) that gave rise to XMRV and are endogenous to some inbred and wild mice. In assessing this situation, it became clear that to rule out false positive results and reliably detect XMRV infection, one must apply several diagnostic methods used in conjunction with known positive and negative controls.

At the NCI-Frederick, we sought to help clarify the XMRV controversy by generating multiple assays, including rigorous methods to measure antibodies to XMRV through ELISA-based methods, to quantify XMRV proviral DNA and viral RNA through quantitative PCR and RT-PCR methods, and to measure infectious virus by viral isolation cultures using an indicator cell line system. We characterized these assays using available positive and negative control samples, including spiked samples and specimens from two pigtail macaques experimentally inoculated with XMRV. We then applied these methods to specimens from two cohorts of prostate cancer patients to determine the levels of XMRV in their blood. Overall, we observed a high level of concordance between detection methods and were able to rule out false positive results by applying multiple assays on the same patient samples. Applying this approach, we did not find evidence of XMRV infection in any of the prostate cancer patient-derived specimens studied.

2. Methods

2.1. Clinical Prostate Cancer Samples. The XMRV detection assays developed at the NCI-Frederick were applied to samples collected from two cohorts of prostate cancer patients. In total, 134 patients were studied. Plasma samples from 108 patients were obtained at the UC Davis Cancer Center. Samples were collected between 2006 and 2010 from prostate cancer patients who were either newly diagnosed, on active treatment, or undergoing post-treatment monitoring. Plasma from all 108 patients was tested for XMRV RNA and antibodies to CA and TM. Institutional Review Board (IRB) approval was obtained from the UC Davis Cancer Center Biorepository, and all study subjects provided written informed consent.

Samples from an additional 26 recently diagnosed prostate cancer patients were obtained from the Urologic Oncology Branch, NIH Clinical Center, Bethesda, MD. All 26 blood samples were tested for the presence of XMRV RNA in plasma and DNA in whole blood. Tests for XMRV proviral DNA were also performed on prostate tissue from 19 of the 26 individuals in this cohort who had radical prostatectomies. Twenty-two of 26 blood samples were tested for antibodies to CA and TM. A subset of 12 samples was tested by virus rescue culture including those that had positive or indeterminate results by X-SCA or ELISA and matched negative controls. The study was approved by the IRB of NCI, NIH, Bethesda, MD, and all study subjects provided written informed consent.

2.2. XMRV Nucleic Assay Detection with XMRV Single-Copy Assays (X-SCA). Similar to the single-copy assay (SCA) for human immunodeficiency virus (HIV) [27], quantitative real-time PCR and RT-PCR assays for detection of XMRV, called XMRV single-copy assays (X-SCA), were developed to quantify XMRV nucleic acid in plasma, whole blood, and cell suspensions obtained from blood or tissue samples. The assays were designed using amplification primers targeting a *gag* leader region conserved between XMRV (as well as PreXMRV-2 [25]) and non-XMRV endogenous MLVs (forward 5-TGTATCAGTTAACCTACCCGAGT-3′, reverse 5-AGACGGGGGCGGGAAGTGTCTC-3′). Consequently, efficient amplification is achieved from both target templates allowing detection of either XMRV or MLVs present in patient samples. The Taqman probe (5′fam-TGG AGT GGC TTT GTT GGG GGA CGA- tamra3′) used for detection of amplified products was designed to span a signature 24 nucleotide deletion in the XMRV (PreXMRV-2) *gag* leader that differentiates these from all other MLV sequences (Figure 1(a)). In the event that a positive sample is identified by X-SCA, single-genome sequencing should be performed to confirm that the source of amplification was XMRV and

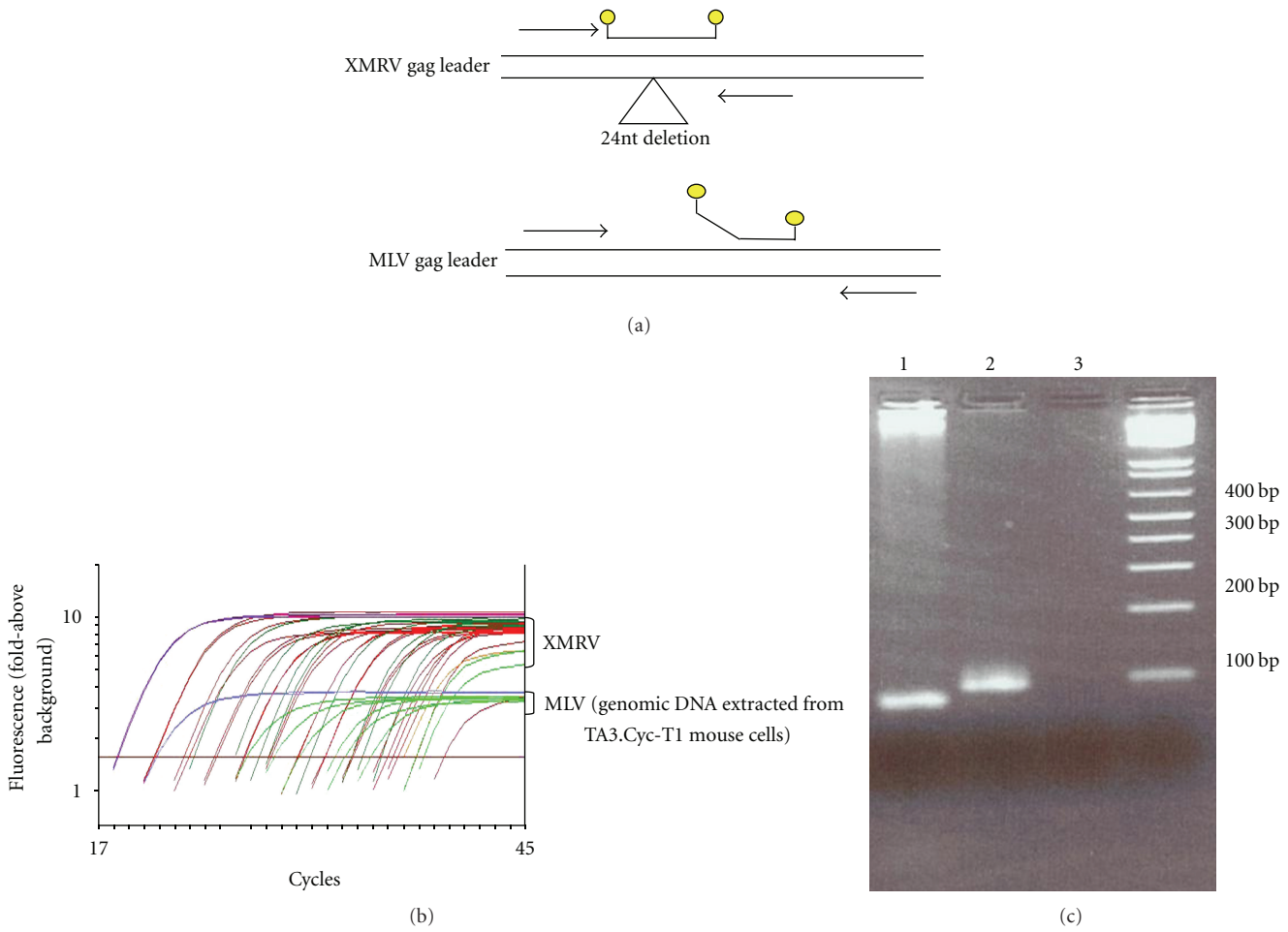


FIGURE 1: *XMRV single-copy assay (X-SCA)*. X-SCA primers anneal to conserved regions in XMRV/MLV gag leader region while the probe spans a 24 nt deletion in XMRV compared to MLV (a) allowing for differential amplification profiles for XMRV and MLV (b). X-SCA amplification products run on a 2% agarose gel distinguish between the products being amplified since the XMRV product is 24 nt smaller than the MLV product. Lane 1 is the X-SCA product from the XMRV standard curve, Lane 2 is the MLV product from the genomic DNA extracted from TA3.Cyc-T1 mouse cells, and Lane 3 is the "no template" negative control (c).

not contaminating mouse DNA with a similar gag deletion, such as PreXMRV-2.

XMRV RNA was extracted from plasma samples following ultracentrifugation exactly as described for HIV SCA [27] and genomic DNA was extracted and whole blood samples using the Promega genomic DNA Extraction Kit (Cat no. A1120) according to the manufacturer's suggested protocol. Reaction conditions for synthesizing cDNA and measuring RNA copy number were exactly as described previously for HIV SCA [27]. XMRV proviral copy number was determined using the Lightcycler 480 Probes Master (Cat no. 04707494001) according to protocol and by performing 45 cycles of 95°C for 15 seconds, 60°C for 1 minute after an initial 10 minute, 95°C polymerase activation step. Accurate detection of XMRV by X-SCA was verified by testing spiked human blood products [28] and by testing blood samples collected from XMRV inoculated macaques (Del Prete et al., in preparation). Pigtail macaques were experimentally inoculated with XMRV (~4.8×10^9 RNA copy equivalents) prepared from the supernatant of 22Rv1 cells (Lot SP1592, Biological Products Core, AIDS and Cancer Virus Program, SAIC-Frederick, Inc, NCI-Frederick). Plasma and PBMC samples were collected prior to inoculation and through 119 days after inoculation. These pre- and post-inoculation specimens were used as reference control samples in evaluating X-SCA methods for detection of XMRV. Details of the macaque infection study will be reported elsewhere (Del Prete et al. in preparation). Animals were housed and cared for in accordance with American Association for Accreditation of Laboratory Animal Care (AALAC) standards in an AAALAC accredited facility, and all animal procedures were performed according to a protocol approved by the Institutional Animal Care and Use Committee of the National Cancer Institute. Detection of MLV was qualified by extracting mouse genomic DNA from TA3.Cyc-T1 cells using the Promega genomic DNA Extraction Kit (Cat no. A1120) and performing X-SCA in duplicate on dilutions of 3000 to 0.03 cell equivalents.

All patient samples were tested by X-SCA in duplicate or triplicate with equal numbers of no template controls (NTC) to monitor the level of false positives due to either viral or mouse genomic DNA contamination. The level of detection for XMRV nucleic acid in clinical samples was determined by the volume of sample available for testing (100 μL to 3 mL). Therefore, X-SCA sensitivity varied from 0.6 to 20.6 copies/mL of plasma and 0.9–10 copies/mL in whole blood. Because of the high frequency of false positives due to contaminating mouse DNA, we set strict criteria for declaring a sample positive for XMRV, requiring detection of viral sequence in all replicate PCR reactions from the samples being tested. These criteria result in a minimum detection of 1.8–41.2 copies XMRV RNA/mL in plasma and 2.7–30 copies XMRV DNA/mL in whole blood for a positive X-SCA test, depending on the volume of sample being tested. If discordant results are obtained from duplicate or triplicate wells, then the result is considered indeterminate and is repeated where sufficient sample is available.

2.3. XMRV Serology. XMRV antigens were prepared in the Protein Expression Laboratory, SAIC-Frederick, MD, as previously described [29]. Purified XMRV antigens were used to develop and optimize ELISA-based protocols (Bagni et al., in preparation). Briefly, purified CA and TM were spotted onto Meso Scale Discovery (MSD) (Gaithersburg, MD) standard 96-well plates at 8 μg/mL and 2 μg/mL, respectively. Samples were diluted 1 : 100 and incubated with individual XMRV antigens. Human antibodies were detected using biotin labeled anti-human IgG (Jackson ImmunoResearch, West Grove, Pa) and MSD-proprietary Sulfo-tagged streptavidin detection reagent and read on a SECTOR Imager 6000 (MSD) plate reader. The XMRV serology assays were qualified with samples obtained from XMRV-inoculated macaques (Del Prete et al., in preparation). Patient samples were considered reactive if the MSD electrochemiluminescent signal (ECL) was at least 50% relative to the ECL signal of the macaque positive control sera. Less reactive patient samples that were at least 2 standard deviations above the average negative human sample were considered indeterminate.

2.4. XMRV Culture Detection. The presence of replication-competent XMRV was determined in a virus rescue coculture assay using indicator cells designated DERSE (Detectors of Exogenous Retroviral Sequence Elements) and using expression of a GFP reporter as the readout. DERSE.LiGP cells are a subclone of LNCaP cells (gift from Dr. Francis Ruscetti, NCI) stably transfected with pBabe.iGFP-puro and screened for susceptibility to XMRV infection (Lee et al., in preparation). pBabe.iGFP-puro is an MLV proviral vector that encodes an intron-interrupted reporter GFP gene and is only expressed after mobilization by an infecting gammaretrovirus for a second round of infection of DERSE.LiGP cells. Similar MLV vectors that only express a reporter after being propagated in infection have been described previously using HEK293 cells [30]. The DERSE.LiGP assay will detect any MLV-related viruses that are capable of replicating in human prostate cancer cells. Virus replication can be detected by monitoring GFP-positive cells either by fluorescence microscopy or FACS analysis.

DERSE.LiGP indicator cells were maintained in Roswell Park Memorial Institute (RPMI) media 1640 (Invitrogen) supplemented with 15% fetal bovine serum (FBS) (Hyclone), 1x Pen/Strep/Glutamine (100 U/mL Penicillin, 0.1 mg/mL Streptomycin, and 0.292 mg/mL Glutamine, Invitrogen) and 1 μg/mL puromycin (Calbiochem). DERSE.LiGP cells were plated at 1×10^5 cells/well in a 24-well tissue culture plate one day before infection. As a positive control, 22Rv1 cell supernatants were diluted in RPMI media and added to cells the next day in the presence of 5 μg/mL of polybrene [31]. Culture medium was refreshed the following day by replacement or splitting cells at a 1 : 3 ratio depending on cell density. Although GFP can be detected in positive control samples within 3 days of infection, to maximize sensitivity for detection of low levels of virus, DERSE.LiGP cells exposed to clinical specimens were maintained in culture for at least two weeks and observed at intervals by fluorescence microscopy. After two weeks, cells were resuspended in a 2% paraformaldehyde (PFA) solution and GFP expression was measured by FACS (FACSCalibur, Becton Dickinson), indicative of a spreading infection. While DERSE.LiGP cells are relatively insensitive to heparin, plasma samples containing EDTA are toxic to the cultures. To mitigate toxicity, 200 μL of EDTA containing plasma samples were distributed into Eppendorf tubes in the presence of 7.5 mM $CaCl_2$ to neutralize the EDTA and 30 U/mL heparin salt to minimize sample clotting. Tubes were incubated for 4 hrs at 4°C to separate the plasma from residual clotting. Accurate detection of XMRV by virus culture was verified using a dilution series of supernatants from 22Rv1 cells and XMRV-spiked human plasma samples containing approximately 10^7 to 10 copies of XMRV RNA. Using XMRV-spiked samples, we noted a loss of detection sensitivity of three- to fivefold in EDTA containing plasma samples treated in the above manner. A recent report of XMRV inactivation by human complement may explain in part the loss of infectivity after addition of plasma [24]. Prostate cancer samples with indeterminate results by X-SCA or ELISA were matched with negative samples and tested blinded in the virus culture assay.

We required that samples test positive for XMRV nucleic acid (RNA or DNA) and by at least one other detect method (immunoassay or culture assay) to be declared positive for XMRV infection.

All reagents developed at the NCI-Frederick and described here are being made available to the extramural research community through the NIH AIDS Research and Reference Reagent Program or AIDS and Cancer Virus Program, SAIC-Frederick, Inc., National Cancer Institute, Frederick.

3. Results

3.1. Differentiating between XMRV and MLV with X-SCA Probe. The X-SCA probe used for detection of amplified products spans a signature 24 nucleotide deletion in the XMRV [1] and in the PreXMRV-2 [32] *gag* leader that

differentiates these from all other MLV sequences (Figure 1(a)). Amplifications of XMRV from 22Rv1 DNA and MLV from mouse genomic DNA (extracted from TA3.CycT1 cells) show that the probe design results in a lower level of plateau fluorescence from non-XMRV MLV templates than from XMRV templates (Figure 1(b)), likely due to inefficient binding and/or degradation of the probe during MLV extension compared to XMRV extension. The result of the probe design is differential amplification profiles for XMRV and MLV, indicating which product is being detected in the assay and the proportions of each if both templates are detected. To confirm the result, the products were run on an agarose gel (Figure 1(c)). The XMRV X-SCA product is 86 nt long and the MLV product 110 nt, easily distinguishable on a 2% agarose gel.

3.2. Qualifying XMRV Assay Detection Capabilities with Spiked Human Samples. Assays for detection of XMRV nucleic acid and replication-competent virus were established using XMRV-spiked samples as positive control specimens. To determine the accuracy and sensitivity of X-SCA methods to detect XMRV in human blood products, we tested a full panel of plasma and whole blood samples that were spiked or not spiked with XMRV derived from 22Rv1 cells. The panel was blinded as to which samples were XMRV positive and which were XMRV negative and were provided to us by the XMRV Scientific Research Working Group for testing by X-SCA [28]. Results from the blinded panel of spiked samples were described previously by Simmons et al. [28] and demonstrated that we detected XMRV RNA and proviral DNA using X-SCA with 100% accuracy. The level of sensitivity for detecting XMRV RNA in the spiked plasma panel was limited by the volume of sample tested for XMRV (270 μL) to 3.3 RNA copies/mL. The level of sensitivity for detecting XMRV proviral DNA was a single XMRV-infected 22Rv1 cell in whole blood samples. All unspiked samples were properly reported as negative for XMRV detection indicating a very low rate of false positivity.

The use of DERSE.L-iG-P cells to detect XMRV was verified using 22Rv1 culture supernatants and XMRV-spiked human plasma. Figure 2 shows the results from virus rescue experiments performed under the following conditions (i) 22Rv1 supernatant alone, (ii) 22Rv1 supernatant treated with $CaCl_2$ and heparin, (iii) 22Rv1 supernatant spiked into human plasma treated with $CaCl_2$ and heparin. DERSE.LiGP cells treated with EDTA-containing human plasma alone are not viable. Proportions of GFP-positive cells detected by FACS at day 4 and day 8 after infection are shown in Figures 2(a) and 2(b). DERSE.LiGP cells exposed to 0.01 μL of 22Rv1 supernatant were GFP-positive by microscopy within 4 days of infection (Figure 2) demonstrating the sensitivity of this assay for detection of replication competent XMRV. The sensitivity of this detection decreased 3–5-fold in the presence of EDTA-containing plasma samples treated as described above. This decrease could in part be due to the presence of human complement as has been recently reported [24]. Additional days of culture increased the number of GFP-positive cells exposed to virus in the presence or absence of plasma. For this reason, cultures infected with human specimens were carried out for a minimum of two weeks.

Table 1: X-SCA Results on XMRV-inoculated macaques.

Monkey ID	Days after inoculation	Plasma XMRV RNA copies/mL	Copies of XMRV DNA per 10^6 PBMCs
14232	0	<1.1	0
14232	5	534.11	197
14232	119	<1.1	23
8242	0	<1.1	0
8242	13	2153.56	2833
8242	119	<1.1	645

Table 2: Immunoassay of plasma from XMRV-inoculated macaques.

Monkey ID	Days after inoculation	Reactivity with	
		CA	TM
8242	0	19.5	248.5
8242	76	12713	544405
14232	0	14.5	145
14232	76	20108	285277

3.3. Verifying Assay Detection Capabilities with Blood Samples from XMRV-Inoculated Macaques. To validate the specificity of X-SCA and ELISA, we used specimens from two pigtail macaques experimentally inoculated with XMRV. Detailed results from the macaque study will be reported elsewhere (Del Prete et al., in preparation). In short, samples tested by X-SCA revealed that peak viremia was achieved at 5 days after inoculation in one animal and at 13 days in the second (Table 1). By day 28, levels of XMRV RNA in plasma had declined to <1 copy/mL in both animals. PBMC-associated XMRV DNA was also measured by X-SCA. DNA levels peaked with similar kinetics as plasma viremia but persisted with levels of 23 and 645 copies/10^6 PBMC in the two animals, respectively, at the end of the follow-up period, 119 days after inoculation. Antibody reactivity to XMRV capsid (CA) and transmembrane protein (TM) measured by ELISA was undetectable prior to inoculation but were robustly positive thereafter (Table 2) (Del Prete et al., in preparation). Replication competent XMRV cannot be cultured from macaque plasma or PBMC samples due to extensive hypermutation of the provirus post-inoculation, likely due to the effect of APOBEC proteins (Del Prete et al., in preparation). Consequently, XMRV-spiked human plasma was used to verify the DERSE.L-iG-P cells for detection of XMRV.

3.4. Testing Prostate Cancer Samples for XMRV Nucleic Acid, Antibodies, and Isolatable Virus. Samples obtained from the two cohorts of prostate cancer patients were assayed first for XMRV nucleic acid (X-SCA) and antibody reactivity against XMRV CA and TM protein (Tables 3 and 4). No

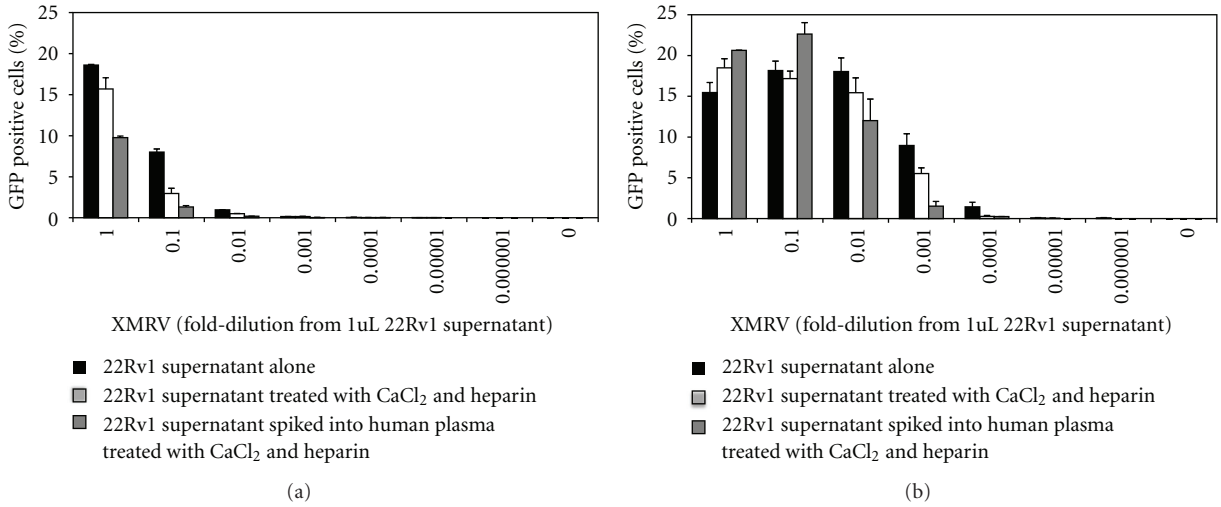


Figure 2: *Verifying XMRV rescue by culturing on DERSE cells with 22Rv1 supernatants and with XMRV-spiked human plasma.* XMRV culturing under the following conditions: (i) 22Rv1 supernatant alone (black bars), (ii) 22Rv1 supernatant treated with $CaCl_2$ + heparin (white bars), (iii) 22Rv1 supernatant spiked into human plasma treated with $CaCl_2$ + heparin (gray bars). GFP-positive cells were analyzed by FACS at day 4 (a) and day 8 (b).

plasma or prostate tissue samples in the NIH prostate cancer cohort or the UC Davis prostate cancer cohort were positive for XMRV nucleic acids or antibodies (Tables 3 and 4). However, two plasma samples in the NIH cohort (0594, 0771) were indeterminate for XMRV RNA. One of these samples (0594) was negative by ELISA, and the other (0771) had an indeterminate ELISA result. One other patient sample in the NIH cohort (0781) was indeterminate for XMRV antibody reactivity but negative for XMRV nucleic acid (Table 3). All three of these samples, along with 9 matched negative samples, were blinded and tested for replicating virus using the DERSE.L-iG-P assay. Virus could not be cultured from any of these plasma samples while it was readily recovered from positive control samples (22Rv1-derived XMRV spiked into negative human plasma) (Figure 3). Consequently, by our prospectively defined criteria, none of the 26 patient samples in the NIH cohort were considered to be XMRV infected (positive for nucleic acid, antibody, and/or replication competent virus) (Table 3). All 108 plasma samples from prostate cancer patients obtained from UC Davis were assayed for XMRV RNA and antibodies (Table 4). All samples were negative for XMRV nucleic acid except one (0739), which was indeterminate. No sample was found to be antibody reactive by our ELISA criteria (at least 50% reactive relative to the macaque positive control sera). Twelve of the 108 samples were indeterminate for XMRV reactivity to either CA or TM (2 standard deviations above the average negative human sample) but were negative for nucleic acid (Table 4). No sample was indeterminate or positive for both XMRV nucleic acid and antibody, and therefore, all were determined to be negative for XMRV infection.

4. Discussion

After publication of the XMRV study by Lombardi et al. in October 2009 suggesting a possible disease association with CFS and a surprisingly high apparent seroprevalence for XMRV even among healthy control subjects, researchers at the NCI-Frederick set out to develop rigorous methods to evaluate the prevalence of XMRV infection. Using control samples, including spiked specimens where appropriate, we developed assays to measure plasma XMRV RNA viremia, cell-associated XMRV DNA levels, and antibodies to XMRV CA and TM. Because Lombardi et al. reported the presence of culture rescuable replication-competent virus from the blood of study subjects using coculture with a human cell line (LNCap), we created DERSE cells, derivatives of the same LNCap cells with a fluorescent reporter to detect XMRV replication. These cells broadly and sensitively detect the replication of different MLV-related gammaretroviruses that exhibit a tropism for human prostate cancer cells. In the absence of patient-derived definitive positive and negative control specimens, we applied our different assay methods to samples obtained from two pigtail macaques prior to and after experimental XMRV inoculation. XMRV plasma viremia was detectable in both inoculated macaques for 2-3 weeks after inoculation but then declined to undetectable levels (Del Prete et al., in preparation). However, XMRV DNA in PBMCs and serum antibodies remained at readily measurable levels for the duration of study follow-up in both animals (Del Prete et al., in preparation). Evaluation of samples from the inoculated macaques demonstrated the ability of our methods to reliably detect evidence of XMRV infection in blood samples and showed that XMRV provirus and antibodies persist even when viremia is not detectable.

In the development of diagnostic tools for XMRV infection, it became clear that a single method for XMRV detection would not be sufficient for definitive diagnosis due to a high frequency of false positives by PCR from contaminating nucleic acids (especially mouse genomic DNA) and high background reactivity seen by ELISA, even in samples

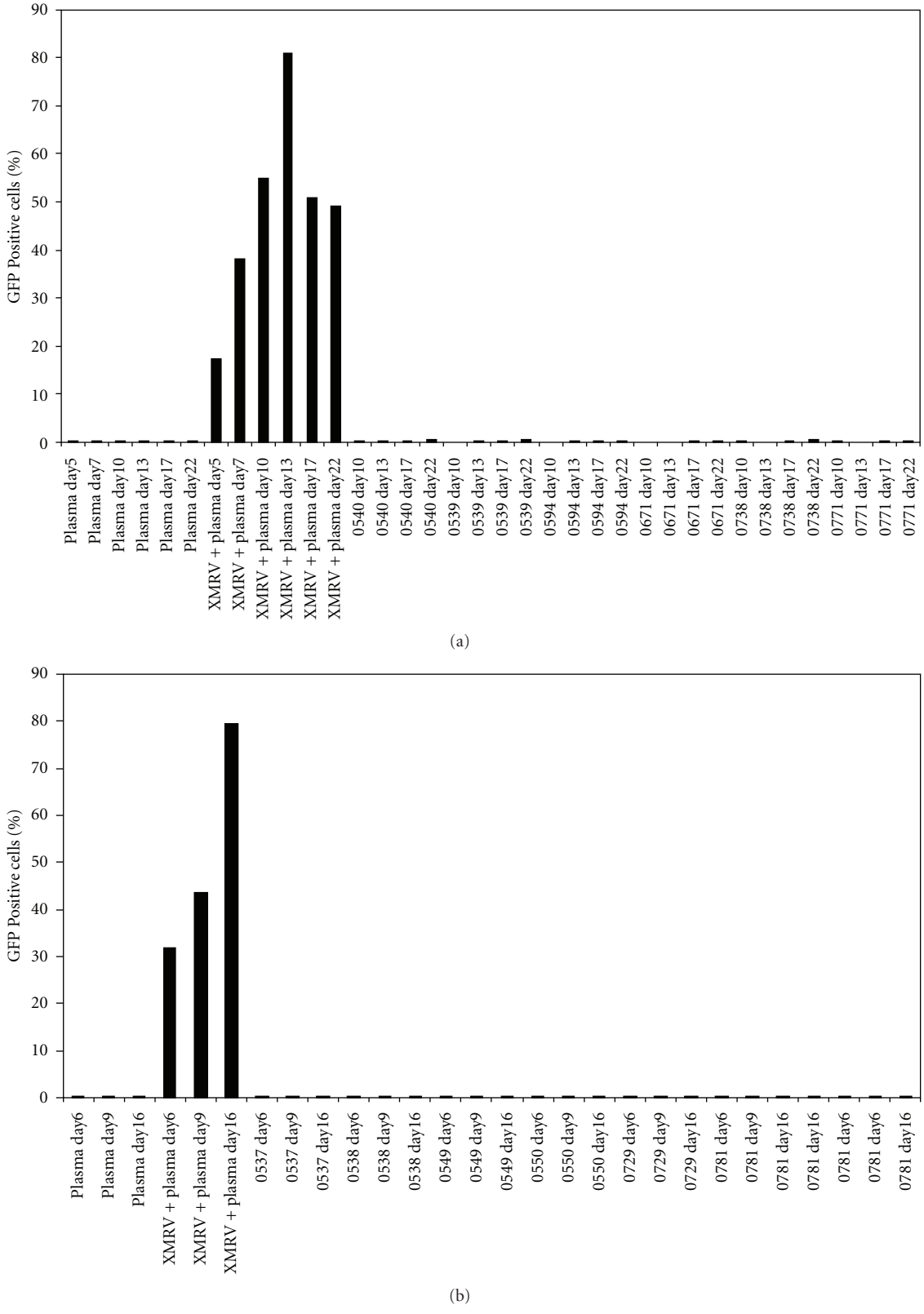


Figure 3: *Testing plasma samples from prostate cancer patients for replication competent XMRV.* Twelve samples were blinded as to their X-SCA and ELISA results and were tested for replicating virus using the DERSE.L-iG-P assay in two separate experiments. Six samples were tested in experiment 1 at passages 10, 13, 17, and 22 (a). All passages were negative for XMRV while virus was recovered from the positive control samples (10^7 copies of XMRV from 22Rv1 cells spiked into human plasma). Six additional samples were tested in experiment 2 at passages 6, 9, and 16 (b). All passages were negative for XMRV while virus was recovered from the positive control samples.

Table 3: X-SCA, ELISA, and virus culture results on prostate cancer samples from NIH cohort.

			Nucieic acid testing by X-SCA				Serologic Testing by ELISA			Virus Culture	
Sample number	Sample ID	Date of blood draw	Plasma XMRV RNA copies/mL	XMRV DNA copies/mL in whole blood	XMRV DNA copies in prostate tissue	Number of prostate cells tested	CA	TM	ELISA result	Virus replication	Overall result
1	UB10-0533	8/5/2010	<0.6	<10	0	174000	999	985	NR	NT	NEGATIVE
2	UB10-0537	8/6/2010	<0.8	<10	NA	NA	5713	5302	NR	NEGATIVE	NEGATIVE
3	UB10-0538	8/6/2010	<0.7	<10	NA	NA	2323	2362	NR	NEGATIVE	NEGATIVE
4	UB10-0539	8/6/2010	<0.8	<0.9	NA	NA	1505	1864	NR	NEGATIVE	NEGATIVE
5	UBI0-0540	8/6/2010	<0.8	<0.9	3.8	105600	1429	2811	NR	NEGATIVE	NEGATIVE
6	UB10-0542	8/9/2010	<0.7	<10	0	307500	1796	1949	NR	NT	NEGATIVE
7	UBI0-0547	8/11/2010	<0.8	<10	0	81000	2248	4325	NR	NT	NEGATIVE
8	UBI0-0548	8/11/2010	<0.8	<10	NA	NA	2566	2826	NR	NT	NEGATIVE
9	UBI0-0549	8/11/2010	<0.8	<10	0	59025	5129	6059	NR	NEGATIVE	NEGATIVE
10	UB10-0550	8/11/2010	<0.8	<10	0	54825	1412	1414	NR	NEGATIVE	NEGATIVE
11	UB10-0578	8/19/2010	<0.8	<10	0	275250	1,412	1,460	NR	NT	NEGATIVE
12	UB10-0594	8/21/2010	*0.9*	<0.9	0	144075	3,050	3,190	NR	NEGATIVE	NEGATIVE
13	UB10-0643	9/14/2010	<0.7	<10	0	32400	5.359	5.430	NR	NT	NEGATIVE
14	UB10-0665	9/16/2010	<0.7	<10	0	84300	2.736	3.817	NR	NT	NEGATIVE
15	UBl0-0671	9/21/2010	Invalid test	<0.9	NA	NA	1,490	1,375	NR	NEGATIVE	NEGATIVE
16	UB10-0706	9/28/2010	Invalid test	<10.0	0	3502.5	2.514	2.500	NR	NT	NEGATIVE
17	UB10-0729	9/30/2010	<0.7	<10.0	0	88950	1,453	1,620	NR	NEGATIVE	NEGATIVE
18	UB10-0771	10/14/2010	*10.4*	<0.9	NA	NA	10.655	26.030	*Equiv*	NEGATIVE	Indeterminate
19	UBl0-0781	10/15/2010	<0.7	<10	NA	NA	8.151	8.451	*Equiv*	NEGATIVE	NEGATIVE
20	UB10-0738	10/5/2010	Invalid test	<0.9	0	74325	2.280	2.312	NR	NEGATIVE	NEGATIVE
21	UB10-0785	10/18/2010	<16.5	<10	0	27375	3.840	3.244	NR	NT	NEGATIVE
22	UB10-0788	10/19/2010	<16.5	<10	0	20887	2.990	2.596	NR	NT	NEGATIVE
23	UBl0-0830	10/29/2010	<0.8	<10	0	63225	NT	NT	NT	NT	NEGATIVE
24	UB10-0833	11/1/2010	<0.8	<10	0	41175	NT	NT	NT	NT	NEGATIVE
25	UBl0-0853	11/4/2010	<0.8	<10	0	8535	NT	NT	NT	NT	NEGATIVE
26	UB10-0913	11/19/2010	<0.8	<10	0	7132	NT	NT	NT	NT	NEGATIVE

NA: sample not available.
NT: sample not tested.

Table 4: X-SCA and ELISA results on prostate cancer samples from UC-Davis cohort.

	Plasma RNA				Plasma RNA		
Patient ID	Copies/mL	ELISA result	Overall result	Patient ID	Copies/mL	ELISA result	Overall result
P0005	<16.5	*Indeterminate*	NEGATIVE	P0566	<16.5	NR	NEGATIVE
P0013	<16.5	NR	NEGATIVE	P0572	<16.5	NR	NEGATIVE
P0015	<16.5	NR	NEGATIVE	P0592	<16.5	NR	NEGATIVE
P0024	<16.5	NR	NEGATIVE	P0593	<16.5	NR	NEGATIVE
P0026	<16.5	NR	NEGATIVE	P0605	<16.5	NR	NEGATIVE
P0027	<16.5	NR	NEGATIVE	P0611	<16.5	NR	NEGATIVE
P0031	<16.5	NR	NEGATIVE	P0612	<16.5	NR	NEGATIVE
P0034	<16.5	NR	NEGATIVE	P0617	<16.5	NR	NEGATIVE
P0036	<16.5	NR	NEGATIVE	P0632	<16.5	NR	NEGATIVE
P0044	<16.5	NR	NEGATIVE	P0637	<16.5	NR	NEGATIVE
P0045	<16.5	NR	NEGATIVE	P0641	<16.5	NR	NEGATIVE
P0118	<16.5	NR	NEGATIVE	P0650	<16.5	NR	NEGATIVE
P0133	<16.5	NR	NEGATIVE	P0657	<16.5	*Indeterminate*	NEGATIVE
P0144	<16.5	NR	NEGATIVE	P0659	<16.5	NR	NEGATIVE
P0154	<16.5	NR	NEGATIVE	P0672	<16.5	NR	NEGATIVE
P0156	<16.5	NR	NEGATIVE	P0673	<16.5	NR	NEGATIVE
P0162	<16.5	*Indeterminate*	NEGATIVE	P0675	<16.5	NR	NEGATIVE
P0167	<16.5	NR	NEGATIVE	P0679	<16.5	NR	NEGATIVE
P0170	<16.5	NR	NEGATIVE	P0685	<16.5	NR	NEGATIVE
P0172	<16.5	NR	NEGATIVE	P0710	<16.5	NR	NEGATIVE
P0177	<16.5	NR	NEGATIVE	P0721	<16.5	NR	NEGATIVE
P0185	<16.5	NR	NEGATIVE	P0723	<20.6	NR	NEGATIVE
P0195	<16.5	*Indeterminate*	NEGATIVE	P0726	<16.5	*Indeterminate*	NEGATIVE
P0209	<16.5	NR	NEGATIVE	P0733	<16.5	NR	NEGATIVE
P0219	<16.5	*Indeterminate*	NEGATIVE	P0739	*55*	NR	NEGATIVE
P0232	<16.5	NR	NEGATIVE	P0766	<20.6	NR	NEGATIVE
P0239	<16.5	NR	NEGATIVE	P0778	<16.5	NR	NEGATIVE
P0293	<16.5	NR	NEGATIVE	P0787	<16.5	NR	NEGATIVE
P0306	<16.5	NR	NEGATIVE	P0792	<16.5	NR	NEGATIVE
P0314	<16.5	NR	NEGATIVE	P0826	<16.5	NR	NEGATIVE
P0321	<16.5	NR	NEGATIVE	P0846	<16.5	NR	NEGATIVE
P0322	<16.5	NR	NEGATIVE	P0848	<16.5	NR	NEGATIVE
P0325	<16.5	NR	NEGATIVE	P0852	<20.6	NR	NEGATIVE
P0327	<16.5	NR	NEGATIVE	P0906	<16.5	*Indeterminate*	NEGATIVE
P0332	<16.5	*Indeterminate*	NEGATIVE	P0916	<16.5	NR	NEGATIVE
P0340	<16.5	*Indeterminate*	NEGATIVE	P0923	<16.5	NR	NEGATIVE
P0342	<16.5	NR	NEGATIVE	P0952	<16.5	NR	NEGATIVE
P0346	<16.5	*Indeterminate*	NEGATIVE	P0984	<16.5	NR	NEGATIVE
P0348	<16.5	NR	NEGATIVE	P0989	<16.5	NR	NEGATIVE
P0351	<16.5	NR	NEGATIVE	P0996	<16.5	NR	NEGATIVE
P0355	<16.5	NR	NEGATIVE	P0999	<16.5	NR	NEGATIVE
P0366	<20.6	NR	NEGATIVE	P1010	<16.5	NR	NEGATIVE
P0380	<16.5	NR	NEGATIVE	P1025	<16.5	NR	NEGATIVE
P0382	<16.5	NR	NEGATIVE	P1032	<16.5	NR	NEGATIVE
P0384	<16.5	NR	NEGATIVE	P1063	<16.5	NR	NEGATIVE

Table 4: Continued.

	Plasma RNA				Plasma RNA		
Patient ID	Copies/mL	ELISA result	Overall result	Patient ID	Copies/mL	ELISA result	Overall result
P0388	<16.5	NR	NEGATIVE	P1076	<16.5	NR	NEGATIVE
P0509	<16.5	*Indeterminate*	NEGATIVE	P1086	<16.5	NR	NEGATIVE
P0511	<16.5	NR	NEGATIVE	P1108	<16.5	*Indeterminate*	NEGATIVE
P0530	<16.5	NR	NEGATIVE	P1110	<16.5	NR	NEGATIVE
P0532	<16.5	NR	NEGATIVE	P1211	<16.5	NR	NEGATIVE
P0535	<16.5	NR	NEGATIVE	P1268	<16.5	NR	NEGATIVE
P0536	<16.5	NR	NEGATIVE	P1297	<16.5	NR	NEGATIVE
P0544	<16.5	NR	NEGATIVE	P1304	<16.5	NR	NEGATIVE
P0562	<16.5	NR	NEGATIVE	P1318	<16.5	NR	NEGATIVE

from healthy control subjects, presumably reflecting cross-reactivity. Therefore, we suggest a multiple assay approach to determine the XMRV status of patient samples. We established diagnostic criteria requiring that all replicates from X-SCA analysis must be positive and that serum antibodies and/or replicating virus must also be detectable in the same patient in order to report the patient XMRV positive. Samples resulting in discordant results from PCR replicates are reported as indeterminate. Despite earlier reports that evidence of XMRV infection was detected in as many as 20% of prostate tumors [2, 10–12], using the assays we developed, we did not find clear evidence for XMRV in the blood of two independent cohorts of patients with prostate cancer (total $n = 134$) or in the prostate tissue of a small subset of these individuals ($n = 19$). Based on previously reported frequencies of XMRV detection in prostate cancer patients, if XMRV is present in the blood of infected individuals, we expected that approximately 27 of the 134 patients in our study would be positive for XMRV. One patient from the NIH cohort (0771) had an indeterminate X-SCA result (2/3 reactions were positive for RNA). This sample was also positive for reactivity to CA and TM by ELISA. However, no XMRV DNA was found in the whole blood from this patient, and replication competent virus could not be recovered from the sample. Taken together, these data are considered an indeterminate result by our criteria. No other samples were positive by more than one diagnostic method.

The occasional positive X-SCA reaction is not above background for this assay. We regularly run 96-well plates of "no template controls" using both our X-SCA primers and primers targeting intracisternal A particles (IAP) [20, 21, 33] that are present in high copies in the mouse genome in order to monitor the levels of contaminating mouse DNA in the reagents and in the environment. We have found that about 5% of wells are positive with the X-SCA primers and about 20% with the IAP primers. Based on these backgrounds, we expect to detect low levels of mouse DNA contamination in samples tested, as seen is this study and in others [20, 21, 33]. Therefore, we required that all replicates of patient samples be positive to obtain a "positive" X-SCA result. We did not test the samples directly with IAP primers since we have not successfully found reagents and an environment that are free from mouse genomic DNA (on average about 1/3000 of a mouse genome per PCR reaction).

Although we had an occasional indeterminate result for XMRV RNA in the plasma samples studied, we did not detect XMRV DNA in any sample tested, despite the ability of our assay to sensitively detect XMRV DNA in spiked control samples and in specimens from inoculated macaques [28] (Del Prete et al., in preparation). Results from the inoculated macaques showed that in experimental infection, XMRV proviral DNA is readily measurable in blood cells even when plasma viremia was not detectable (Del Prete et al., in preparation), further suggesting that these patients do not carry XMRV in their blood. Findings from previous studies reporting higher prevalence for XMRV in similar cohorts [2, 11, 12] typically involved testing of prostate tumors. None of these studies reported the detection of XMRV in blood samples or the isolation of infectious virus from clinical specimens, and only one measured the presence of reactive antibodies through a virus neutralization assay [10]. Detection of antibody responses to specific viral proteins by ELISA or by reactivity to XMRV immunoblots was not assessed. If we had used less rigorous criteria basing an overall diagnosis on a single, nonconfirmed test and not requiring all replicates to yield the same result, then our two cohorts would have given rise to an apparent, and in our view almost certainly incorrect, reported XMRV prevalence rate of approximately 12%. These considerations may explain conflicting prior reports for the prevalence of XMRV and are consistent with claims that XMRV detection is likely the result of laboratory contamination [22, 26, 33, 34]. Particularly given the potential for false positive results in PCR and serological assays for XMRV, our results suggest that applying multiple diagnostic methods including measuring levels of proviral DNA in blood cells provides a more reliable approach for investigating the prevalence of XMRV. These results also demonstrate that XMRV nucleic acid, and antibodies are undetectable in the blood of patients with prostate cancer.

Acknowledgments

The authors thank Valerie Boltz for helpful discussions and Connie Kinna, Susan Jordan, and Susan Toms for administrative support, and Jeremy Smedley, Rhonda Macallister, and Mercy Gathuka for expert animal care. They thank the XMRV Scientific Research Working Group for providing samples used to verify X-SCA methods. JMC was a Research Professor of the American Cancer Society, with support from the FM Kirby Foundation. Funding for this research was provided by the National Cancer Institute's intramural Center for Cancer Research and Office for AIDS Research and in part with federal funds from the National Cancer Institute under contract HHSN261200800001E. This work was also supported in part by Bench-to-Bedside Award to V. K. Pathak. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the US Government.

References

[1] V. C. Lombardi, F. W. Ruscetti, J. D. Gupta et al., "Detection of an infectious retrovirus, XMRV, in blood cells of patients with chronic fatigue syndrome," *Science*, vol. 326, no. 5952, pp. 585–589, 2009.

[2] A. Urisman, R. J. Molinaro, N. Fischer et al., "Identification of a novel gammaretrovirus in prostate tumors of patients homozygous for R462Q RNASEL variant," *Plos Pathogens*, vol. 2, no. 3, article e25, pp. 211–225, 2006.

[3] J. Carpten, N. Nupponen, S. Isaacs et al., "Germline mutations in the ribonuclease L gene in families showing linkage with HPC1," *Nature Genetics*, vol. 30, no. 2, pp. 181–184, 2002.

[4] G. Casey, P. J. Neville, S. J. Plummer et al., "RNASEL Arg462Gln variant is implicated in up to 13% of prostate cancer cases," *Nature Genetics*, vol. 32, no. 4, pp. 581–583, 2002.

[5] K. Malathi, J. M. Paranjape, E. Bulanova et al., "A transcriptional signaling pathway in the IFN system mediated by 2'-5'-oligoadenylate activation of RNase L," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 41, pp. 14533–14538, 2005.

[6] H. Rennert, D. Bercovich, A. Hubert et al., "A novel founder mutation in the RNASEL gene, 471delAAAG, is associated with prostate cancer in ashkenazi jews," *American Journal of Human Genetics*, vol. 71, no. 4, pp. 981–984, 2002.

[7] A. Rokman, T. Ikonen, E. H. Seppala et al., "Germline alterations of the RNASEL gene, a candidate HPC1 gene at 1q25, in patients and families with prostate cancer," *American Journal of Human Genetics*, vol. 70, no. 5, pp. 1299–1304, 2002.

[8] L. Wang, S. K. McDonnell, D. A. Elkins et al., "Analysis of the RNASEL gene in familial and sporadic prostate cancer," *American Journal of Human Genetics*, vol. 71, no. 1, pp. 116–123, 2002.

[9] R. Schlaberg, D. J. Choe, K. R. Brown, H. M. Thaker, and I. R. Singh, "XMRV is present in malignant prostatic epithelium and is associated with prostate cancer, especially high-grade tumors," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 38, pp. 16351–16356, 2009.

[10] R. S. Arnold, N. V. Makarova, A. O. Osunkoya et al., "XMRV infection in patients with prostate cancer: novel serologic assay and correlation with PCR and FISH," *Urology*, vol. 75, no. 4, pp. 755–761, 2010.

[11] R. Schlaberg, D. J. Choe, K. R. Brown, H. M. Thaker, and I. R. Singh, "XMRV is present in malignant prostatic epithelium and is associated with prostate cancer, especially high-grade tumors," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 38, pp. 16351–16356, 2009.

[12] B. P. Danielson, G. E. Ayala, and J. T. Kimata, "Detection of xenotropic murine leukemia virus-related virus in normal and tumor tissue of patients from the southern United States with prostate cancer is dependent on specific polymerase chain reaction conditions," *The Journal of Infectious Diseases*, vol. 202, no. 10, pp. 1470–1477, 2010.

[13] B. C. Satterfield, R. A. Garcia, H. Jia, S. Tang, H. Zheng, and W. M. Switzer, "Serologic and PCR testing of persons with chronic fatigue syndrome in the United States shows no association with xenotropic or polytropic murine leukemia virus-related viruses," *Retrovirology*, vol. 8, article 12, 2011.

[14] O. Hohn, K. Strohschein, A. U. Brandt et al., "No evidence for XMRV in German CFS and MS patients with fatigue despite the ability of the virus to infect human blood cells in vitro," *Plos One*, vol. 5, no. 12, Article ID e15632, 2010.

[15] M. Cornelissen, F. Zorgdrager, P. Blom et al., "Lack of detection of XMRV in seminal plasma from HIV-1 infected men in The Netherlands," *Plos One*, vol. 5, no. 8, Article ID e12040, 2010.

[16] O. Erlwein, S. Kaye, M. O. McClure et al., "Failure to detect the novel retrovirus XMRV in chronic fatigue syndrome," *Plos One*, vol. 5, no. 1, Article ID e8519, 2010.

[17] E. R. Gray, J. A. Garson, J. Breuer et al., "No evidence of XMRV or related retroviruses in a london HIV-1-positive patient cohort," *Plos One*, vol. 6, no. 3, Article ID e18096, 2011.

[18] S. C. Lo, N. Pripuzova, B. Li et al., "Detection of MLV-related virus gene sequences in blood of patients with chronic fatigue syndrome and healthy blood donors," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 36, pp. 15874–15879, 2010.

[19] M. Kearney and F. Maldarelli, "Current status of xenotropic murine leukemia virus-related retrovirus in chronic fatigue syndrome and prostate cancer: reach for a scorecard, not a prescription pad," *The Journal of Infectious Diseases*, vol. 202, no. 10, pp. 1463–1466, 2010.

[20] B. Oakes, A. K. Tai, O. Cingoz et al., "Contamination of human DNA samples with mouse DNA can lead to false detection of XMRV-like sequences," *Retrovirology*, vol. 7, p. 109, 2010.

[21] M. J. Robinson, O. W. Erlwein, S. Kaye et al., "Mouse DNA contamination in human tissue tested for XMRV," *Retrovirology*, vol. 7, p. 108, 2010.

[22] J. A. Garson, P. Kellam, and G. J. Towers, "Analysis of XMRV integration sites from human prostate cancer tissues suggests PCR contamination rather than genuine human infection," *Retrovirology*, vol. 8, article 13, 2011.

[23] S. Hue, E. R. Gray, A. Gall et al., "Disease-associated XMRV sequences are consistent with laboratory contamination," *Retrovirology*, vol. 7, p. 111, 2010.

[24] K. Knox, D. Carrigan, G. Simmons et al., "No evidence of murine-like gammaretroviruses in CFS patients previously identified as XMRV-infected," *Science*, vol. 333, no. 6038, pp. 94–97, 2011.

[25] T. Paprotka, K. A. Delviks-Frankenberry, O. Cingoz et al., "Recombinant origin of the retrovirus XMRV," *Science*, 2011.

[26] P. W. Tuke, K. I. Tettmar, A. Tamuri, J. P. Stoye, and R. S. Tedder, "PCR master mixes harbour murine DNA sequences. caveat emptor!," *Plos One*, vol. 6, no. 5, 2011.

[27] S. Palmer, A. P. Wiegand, F. Maldarelli et al., "New real-time reverse transcriptase-initiated PCR assay with single-copy sensitivity for human immunodeficiency virus type 1 RNA in plasma," *Journal of Clinical Microbiology*, vol. 41, no. 10, pp. 4531–4536, 2003.

[28] G. Simmons, S. A. Glynn, J. A. Holmberg et al., "The blood xenotropic murine leukemia virus-related virus scientific research working group: mission, progress, and plans," *Transfusion*, vol. 51, no. 3, pp. 643–653, 2011.

[29] W. K. Gillette, D. Esposito, T. E. Taylor, R. F. Hopkins, R. K. Bagni, and J. L. Hartley, "Purify first: rapid expression and purification of proteins from XMRV," *Protein Expression and Purification*, vol. 76, pp. 238–247, 2011.

[30] J. Jin, F. Li, and W. Mothes, "Viral determinants of polarized assembly for the murine leukemia virus," *Journal of Virology*, vol. 85, no. 15, pp. 7672–7682, 2011.

[31] E. C. Knouf, M. J. Metzger, P. S. Mitchell et al., "Multiple integrated copies and high-level production of the human retrovirus XMRV (xenotropic murine leukemia virus-related virus) from 22Rv1 prostate carcinoma cells," *Journal of Virology*, vol. 83, no. 14, pp. 7353–7356, 2009.

[32] T. Paprotka, K. A. Delviks-Frankenberry, O. Cingoz et al., "Recombinant origin of the retrovirus XMRV," *Science*, vol. 333, no. 6038, pp. 97–101, 2011.

[33] B. T. Huber, B. Oakes, A. K. Tai et al., "Contamination of human DNA samples with mouse DNA can lead to false detection of XMRV-like sequences," *Retrovirology*, vol. 7, p. 109, 2010.

[34] J. N. Baraniuk, "Xenotropic murine leukemia virus-related virus in chronic fatigue syndrome and prostate cancer," *Current Allergy and Asthma Reports*, vol. 10, no. 3, pp. 210–214, 2010.

Hindawi Publishing Corporation
Advances in Virology
Volume 2011, Article ID 965689, 5 pages
doi:10.1155/2011/965689

Research Article

Sexual Transmission of XMRV: A Potential Infection Route

Prachi Sharma,[1] Kenneth A. Rogers,[1] Suganthi Suppiah,[2] Ross J. Molinaro,[2] Nattawat Onlamoon,[2,3] John Hackett Jr.,[4] Gerald Schochetman,[4] Eric A. Klein,[5] Robert H. Silverman,[6] and François Villinger[1,2]

[1] *Division of Pathology, Yerkes National Primate Research Center, Emory University, Atlanta, GA 30329, USA*
[2] *Department of Pathology and Laboratory Medicine, Emory University School of Medicine, Atlanta, GA 30329, USA*
[3] *Office for Research and Development, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok, Bangkok 10700, Thailand*
[4] *Abbott Diagnostics, Emerging Pathogens and Virus Discovery, Abbott Park, IL 60064, USA*
[5] *Glickman Urological and Kidney Institute and LRI, Cleveland Clinic Foundation, Cleveland, OH 44195, USA*
[6] *Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, OH 44195, USA*

Correspondence should be addressed to Prachi Sharma, psharm9@emory.edu

Received 27 April 2011; Accepted 25 May 2011

Academic Editor: Arifa S. Khan

Although XMRV dissemination in humans is a matter of debate, the prostate of select patients seem to harbor XMRV, which raises questions about its potential route of transmission. We established a model of infection in rhesus macaques inoculated with XMRV. In spite of the intravenous inoculation, all infected macaques exhibited readily detectable XMRV signal in the reproductive tract of all 4 males and 1 female during both acute and chronic infection stages. XMRV showed explosive growth in the acini of prostate during acute but not chronic infection. In seminal vesicles, epididymis, and testes, XMRV protein production was detected throughout infection in interstitial or epithelial cells. In the female monkey, epithelial cells in the cervix and vagina were also positive for XMRV gag. The ready detection of XMRV in the reproductive tract of male and female macaques infected intravenously suggests the potential for sexual transmission for XMRV.

1. Introduction

Xenotropic murine leukemia virus-related retrovirus (XMRV), a gammaretrovirus, was initially discovered in a study that used prostate carcinoma tissues and later in a study that used blood of chronic fatigue syndrome patients [1, 2], although other labs have been unable to detect XMRV in such patients [3] or have suggested that most of the findings based on nucleic amplification techniques were secondary to contamination with mouse DNA [4–7]. Moreover the link to chronic fatigue has recently been seriously questioned [8]. However, the detection of XMRV in select prostate cancers was not all based on methods using nucleic acid amplification, and while the etiological role of XMRV infection for cancer remains uncertain, XMRV represents a novel gamma retrovirus capable of infecting several human host cell lines [9], and hence, a potential zoonotic agent. In fact, infection of humans with this virus might have resulted from a zoonotic transmission from mouse to man [1] similar to the transspecies transmission reported with the Koala retrovirus, a gammaretrovirus closely related to XMRV [10]. Though both cell-associated and cell-free transmission of XMRV have been reported [2] and there is indirect evidence for potential respiratory [11] or sexual [12] transmission, the exact route or mechanism of transmission still remains unresolved. To better understand the XMRV pathogenesis, our lab recently established an animal model of XMRV infection using rhesus macaques. Results of this study have recently been published, demonstrating unequivocally that XMRV is infectious for primates, inducing a persistent infection which, given the right context, may be reactivated in vivo [13]. During the histological analyses of these animals, it was realized that in spite of the generalized infection, XMRV appeared to show a predilection for tissues and organs of the reproductive tract in this model, suggesting the potential for sexual transmission.

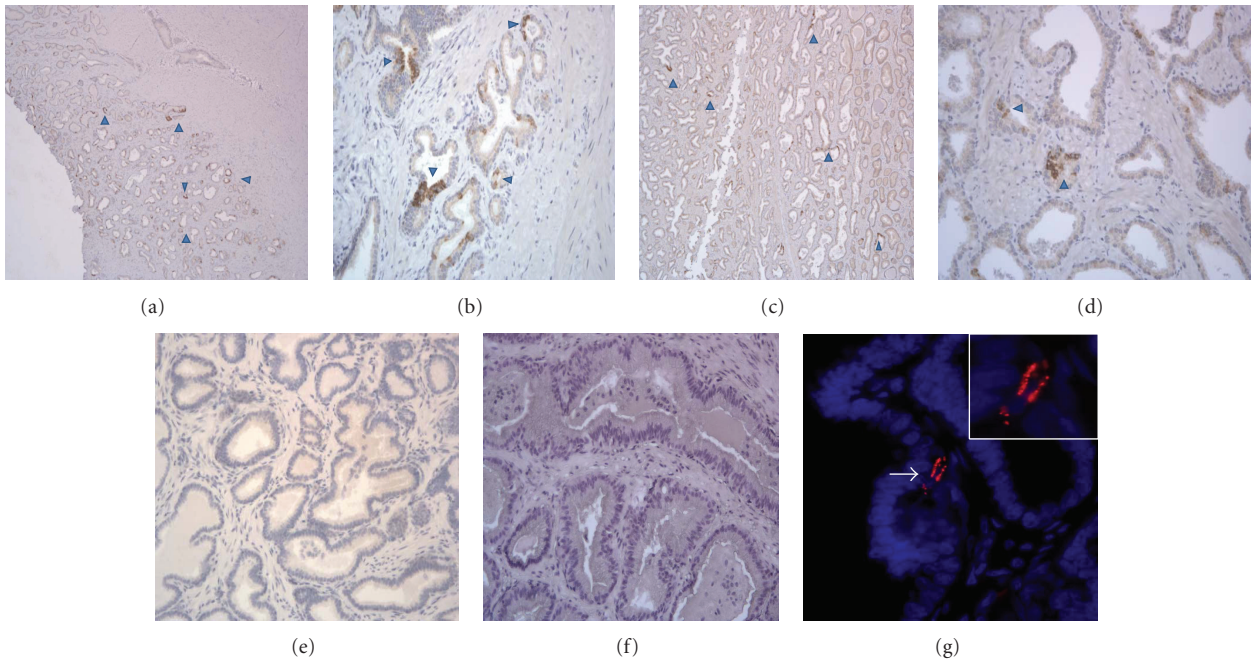


Figure 1: Detection of XMRV gag in the prostates of two acutely infected macaques RLm-1 ((a, b), day 6 pi) and ROu-4 ((c, d), day 7 pi); one chronically infected macaque RLq-10 ((e, f), day 144 pi); control macaque RPd-7 (f). Immunohistochemistry ((a)–(f)). Magnification, ×40 ((a, c)), ×200 ((b, d, e, and f)). FISH (g), magnification, ×600, ×1000.

2. Materials and Methods

2.1. Animals. Nine adult healthy rhesus macaques (5 infected—4 males and 1 female and 4 controls—3 males and 1 female) of Indian origin with ages ranging from 5–17 years were used for this study. These macaques were housed at the Yerkes National Primate Research Center at Emory University under BSL-2+ housing conditions and maintained in accordance with the instructions of the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council, and the U.S. Public Health Service (PHS) Guidelines, *Guide for the Care and Use of Laboratory Animals*.

2.2. XMRV Inoculation. Five monkeys were inoculated with 3.6×10^6 $TCID_{50}$ of XMRV grown in DU145 prostate cancer cells delivered intravenously [13]. Euthanasia and necropsies of the first 3 monkeys were performed at days 6, 7 (acute infection, RLm-1 and ROu-4, resp.), and 144 pi (RLq-10) after a single-XMRV infection. The last 2 animals (RYl-10 and RIm-10) were reinoculated at day 158 after infection (pi) with 3.6×10^6 $TCID_{50}$ of sucrose-purified XMRV (Advanced Biotechnologies, Inc., Columbia, Md, USA) again delivered IV. These 2 monkeys were euthanized at day 291/133 pi (after primary/secondary infection, resp.). At necropsy, reproductive tissue from the males (prostate, testes, seminal vesicle, and epididymis) and females (cervix and vagina) were collected in 10% neutral buffered formalin and fixed for 24 hours. Control animals (three males, one female) were not infected with XMRV, but housed and cared for in similar accommodations, but not in the same rooms. Three of the controls were sacrificed for noninfectious reasons, and one (male) was chronically infected with simian immunodeficiency, but without clinical progression to AIDS.

2.3. Immunohistochemistry. The formalin-fixed paraffin-embedded tissue was sectioned at 4 μM thickness and slides prepared. After deparaffinization, the slides were rehydrated and antigen retrieval done by microwave treatment after quenching of endogenous peroxydases. The sections were incubated with a 1 : 100 dilution of a rat anti-SFFV antibody cross-reactive to XMRV [1] followed by biotinylated antirat polyclonal antibody and the ABC reagent (Vector Laboratories). Virus was detected by the development of the chromogenic substrate 3,3′-diaminobenzidine (Dako) and counterstained with hematoxylin. Each run was performed with relevant negative and positive controls. Slides were read using an Olympus BX-41 light microscope. Fluorescent in situ hybridization (FISH) to XMRV nucleic acids was performed as described previously [1, 13]. Though some recent reports that have suggested XMRV detection using RT-PCR in specimens collected from human patients may be the result of laboratory contamination [4–7], the data generated in our study did not rely on amplification techniques involving PCR.

The techniques used here (IHC and FISH) were not susceptible to contamination errors, our laboratory does not work with rodent tissues, and while tissue collections from each animal were done at different times, embedding and slides preparation were processed as a batch.

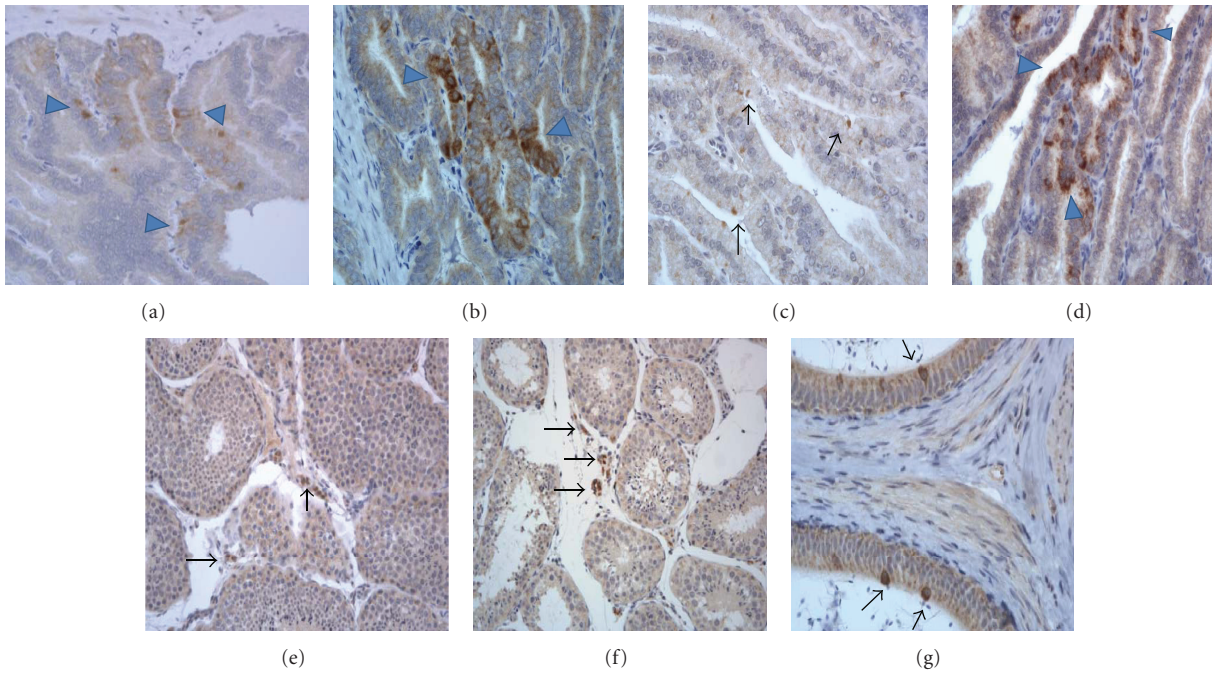


Figure 2: Detection of XMRV gag in the other reproductive tissues of the infected male macaques: seminal vesicle, ROu-4 ((a) day 7 pi), RLm-1 ((b) day 6 pi), RIl-10 ((c) day 291/133 pi), RLq-10 ((d) day 144 pi), testis, RIl-10 ((e) day 291/133 pi), testis, RLq-10 ((f) day 144 pi), epididymis, RLq-10 ((g) day 144 pi). Immunohistochemistry. Magnification, ×400 (a, b, c, d, g) and ×200 (e, f).

3. Results and Discussion

A detailed analysis of the XMRV infection, viral dissemination, and antibody responses in these macaques is published elsewhere [13]. Here, our analyses focused on the viral distribution in the reproductive tract and its potential implication for sexual transmission. Briefly, none of the infected animals showed any obvious clinical symptoms for the entire 9 months of followup, based on activity levels, food intake, hematological, or serum chemistry findings. The infected animals were sacrificed at different times after infection to obtain tissues representative of the acute and chronic virus dissemination with clear separation from one another. Control and XMRV-infected tissues were, however, embedded and sectioned in parallel, and assayed together as batches. All tissues were Lymphoid organs like spleen, lymph nodes, and the gastrointestinal lamina propria and nonlymphoid organs like lung, brain, liver, and bone marrow contained cells positive for XMRV by IHC and/or FISH for which semiquantitative analysis was performed [13]. Of note, several tissues such as CNS, heart, adrenal gland, gall bladder, kidney, and urinary bladder were negative by IHC with only a rare single FISH signal [13]. In contrast, there was an absence of signal all organs of uninfected control rhesus macaques run in the same batches as samples from XMRV infected monkeys.

No significant gross or histologic lesions were observed in the reproductive tract of all animals except for the presence of mononuclear cell infiltrates in prostate tissues [13], although such findings are not uncommon in age matched controls. During acute XMRV infection (days 6-7 pi) extensive foci of

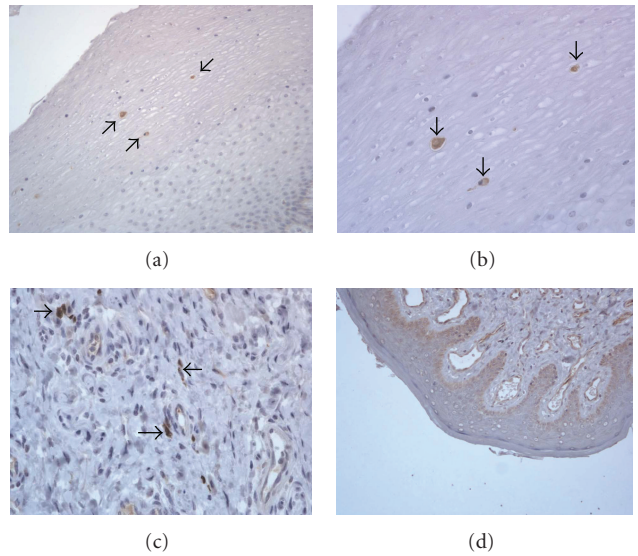


Figure 3: Detection of XMRV gag in the cervix (a, b) and vagina (c) of the female rhesus macaque (RYh-10, day 291/133 pi), and in the vagina of a control female rhesus macaque RFm-2 (d). Magnification, ×200 (a, d), ×400 (b, c).

XMRV, positive acinar epithelial cells were detected in the prostate of these monkeys. (Figures 1(a) and 1(d)). Figures 1(a) and 1(c) (arrows) show an overview of the multiple foci of positively staining glandular epithelial cells in the prostate of the two acutely infected animals. The presence of XMRV in the epithelial cells of the prostate, with the surrounding

stroma being negative during the acute infection, suggests cell-to-cell transmission in the glandular acini and also that prostate is a predilection site for the early XMRV infection. Though XMRV was no longer detected by IHC in the prostate during the chronic phase of infection (Figure 1(e)), similar to the control macaques (Figure 1(f)), XMRV nucleic acid signals were still observed using FISH (Figure 1(g)). This suggests a lack of replication, an unsuitable environment, or some form of active control of viral replication which remains to be determined.

Other tissues showing positive staining in the male-reproductive tract included seminal vesicles (Figure 2(a) and 2(d)) and testes of both acute (Figure 2(e)) and chronically (Figure 2(f)) infected animals. The epididymis of one chronically infected animal (Figure 2(g)) revealed frequent positive epithelial cells. Of interest, the morphology of the cells expressing XMRV protein varied from glandular epithelial cells in the prostate, seminal vesicle, and epididymis to the interstitial cells in the testes. Remarkably, even the reproductive tissue sampled from the only female monkey included in our study showed positive XMRV staining at 9 months after infection. Occasional mucosal epithelial cells in the cervical epithelium (Figures 3(a) and 3(b)) and submucosal cells, presumably fibroblasts, (Figure 3(c)) in the vaginal submucosa expressed XMRV gag protein. Viral protein detection was consistently negative in the reproductive tract of the control female rhesus macaque (Figure 3(d)).

Our study used the intravenous mode of virus administration to ensure infection. But, despite using this route, the virus to our surprise rapidly concentrated in the lower reproductive tract of both male and female macaques, suggesting a potential for the sexual mode of transmission. Lending support to this hypothesis are previous reports showing the detection of XMRV RNA in human prostatic secretions [12], and the finding that XMRV replication is enhanced by androgens due to the presence of a glucocorticoid response element (GRE) [7]. Moreover, human semen or cationic amyloid fibrils, a degradation product from prostatic acid phosphatase, has been shown to promote the transmission of HIV and XMRV in vitro [12, 14, 15], suggesting another mechanism potentially facilitating mucosal transmission in vivo. Though recent reports have suggested that XMRV detection using RT-PCR in specimens collected from human patients may be the result of laboratory contamination [12], the data generated in our study relied on techniques (IHC and FISH) that did not include gene amplification and are therefore rather impervious to sample contamination. Moreover, negative controls on this study were consistently negative using these techniques.

In conclusion, our study demonstrates XMRV protein expression in the reproductive tract of the experimentally infected rhesus macaques at all times after infection supporting the potential for sexual transmission of this virus.

Author Contributions

Eric A. Klien, John Hackett, Robert H. Silverman, and François Villinger designed the study. Prachi Sharma, Suganthi Suppiah, Ross J. Molinaro, Nattawat Onlamoon, Kenneth A. Rogers, and François Villinger performed the research and analyzed the data. Prachi Sharma wrote the paper.

Acknowledgment

These studies were supported by Abbott Diagnostics and the NCRR support to the Yerkes NPRC DRR000165.

References

[1] A. Urisman, R. J. Molinaro, N. Fischer et al., "Identification of a novel gammaretrovirus in prostate tumors of patients homozygous for R462Q RNASEL variant," *PLoS Pathogens*, vol. 2, no. 3, article e25, 2006.

[2] V. C. Lombardi, F. W. Ruscetti, J. D. Gupta et al., "Detection of an infectious retrovirus, XMRV, in blood cells of patients with chronic fatigue syndrome," *Science*, vol. 326, no. 5952, pp. 585–589, 2009.

[3] J. P. Stoye, R. H. Silverman, C. A. Boucher, and S. F. Le Grice, "The xenotropic murine leukemia virus-related retrovirus debate continues at first international workshop," *Retrovirology*, vol. 7, p. 113, 2010.

[4] S. Hue, E. R. Gray, A. Gall et al., "Disease-associated XMRV sequences are consistent with laboratory contamination," *Retrovirology*, vol. 7, no. 1, p. 111, 2010.

[5] B. Oakes, A. K. Tai, O. Cingoz et al., "Contamination of human DNA samples with mouse DNA can lead to false detection of XMRV-like sequences," *Retrovirology*, vol. 7, no. 1, p. 109, 2010.

[6] M. J. Robinson, O. W. Erlwein, S. Kaye et al., "Mouse DNA contamination in human tissue tested for XMRV," *Retrovirology*, vol. 7, no. 1, p. 108, 2010.

[7] E. Sato, R. A. Furuta, and T. Miyazawa, "An endogenous murine leukemia viral genome contaminant in a commercial RT-PCR Kit is amplified using standard primers for XMRV," *Retrovirology*, vol. 7, no. 1, p. 110, 2010.

[8] C. H. Shin, L. Bateman, R. Schlaberg et al., "Absence of XMRV and other murine leukemia virus-related viruses in patients with chronic fatigue syndrome," *The Journal of Virology*, vol. 85, pp. 7195–7202, 2011.

[9] K. Stieler, C. Schulz, M. Lavanya, M. Aepfelbacher, C. Stocking, and N. Fischer, "Host range and cellular tropism of the human exogenous gammaretrovirus XMRV," *Virology*, vol. 399, no. 1, pp. 23–30, 2010.

[10] R. E. Tarlinton, J. Meers, and P. R. Young, "Retroviral invasion of the koala genome," *Nature*, vol. 442, no. 7098, pp. 79–81, 2006.

[11] N. Fischer, C. Schulz, K. Stieler et al., "Xenotropic murine leukemia virus-related gammaretro virus in respiratory tract," *Emerging Infectious Diseases*, vol. 16, no. 6, pp. 1000–1002, 2010.

[12] S. Hong, E. A. Klein, J. Das Gupta et al., "Fibrils of prostatic acid phosphatase fragments boost infections with XMRV (xenotropic murine leukemia virus-related virus), a human retrovirus associated with prostate cancer," *The Journal of Virology*, vol. 83, no. 14, pp. 6995–7003, 2009.

[13] N. Onlamoon, J. D. Gupta, P. Sharma et al., "Infection, viral dissemination and antibody responses of rhesus macaques exposed to the human gammaretrovirus XMRV," *The Journal of Virology*, vol. 85, no. 9, pp. 4547–4557, 2011.

[14] J. Münch, E. Rücker, L. Ständker et al., "Semen-derived amyloid fibrils drastically enhance HIV infection," *Cell*, vol. 131, no. 6, pp. 1059–1071, 2007.

[15] N. R. Roan, J. Münch, N. Arhel et al., "The cationic properties of SEVI underlie its ability to enhance human immunodeficiency virus infection," *The Journal of Virology*, vol. 83, no. 1, pp. 73–80, 2009.