Susceptibility of xenotropic murine leukemia virus-related virus (XMRV) to retroviral restriction factors

Harriet C. T. Groom, Melvyn W. Yap, Rui Pedro Galão, Stuart J. D. Neil, and Kate N. Bishop

In 2006, a unique gammaretrovirus was isolated from patients with familial prostate cancer (1). This virus is highly homologous to several endogenous MLVs found in mice and murine cell lines, and was named xenotropic MLV-related virus (XMRV). In their study, Urisman et al. found that the presence of XMRV RNA correlated with a deficiency in RNase L, a molecule involved in the antiviral response induced by IFN. Subsequent studies have also detected XMRV in cells from prostate cancer patients (2, 3), although these studies have found no correlation with the RNASEL genotype. By contrast, another group looking for XMRV DNA in prostate cancer found no evidence of the virus in their cohort of patients (4). Very recently it has been reported that XMRV DNA is present in 67% chronic fatigue syndrome (CFS) patients, compared with only 3.7% of controls (5). Cell-culture experiments revealed the presence of infectious XMRV that could be passed from patient-derived peripheral blood mononuclear cells (PBMC), T cells, B cells, and cell-free serum to activated primary lymphocytes and other indicator cells (5). At this time, it is unclear whether prostate cancer or CFS is linked to infection by XMRV or, indeed, how prevalent the virus is in the general population (6). If confirmed in further studies, these results could have a major impact on both the diagnosis and potential treatment of both diseases. It is therefore important to understand more about the replication and spread of XMRV.

Over the last few years it has become increasingly apparent that both the species specificity and tissue tropism of retroviruses are influenced by host-encoded innate restriction factors that target various stages of the retroviral replication cycle (7). Three major classes of retroviral restriction factor have so far been identified in mammals: members of the apolipoprotein B mRNA-editing complex (APOBEC) family that target viral nucleic acid (8, 9); Friend-virus susceptibility factor 1 (Fv1) (10, 11) and tripartite motif 5 (TRIM5) family members that inactivate incoming viral capsids after entry (12, 13); and tetherin/BST-2/CD317 that restricts the release of nascent retroviral particles from infected cells (14, 15). Several human restriction factors are able to inhibit MLV replication. For example, human APOBEC3G (hA3G) restricts Moloney (Mo)-MLV (16–19), human TRIM5α blocks N-tropic MLV (20–23), and tetherin potently inhibits the release of gammaretroviral particles from transfected cells (14, 24). Because all of these factors can be expressed in peripheral blood lymphocytes, to replicate in these cells as reported (5) XMRV may have evolved means to evade or overcome these proteins. It is worth noting that lentiviruses, like HIV, express accessory genes that counteract different restriction factors and thus protect the virus from inhibition (25). However, XMRV, like MLV, does not encode such accessory genes and would not be expected to overcome restriction by canonical means. Determining the restriction pattern of XMRV will facilitate the identification of the potential target cells for the virus and will allow infection to be related to pathology. It is also important to understand the effects of restriction factors on XMRV when considering nonhuman reservoirs of viral replication and when designing animal models of diseases associated with the virus.

We therefore set out to evaluate whether XMRV infection is inhibited by a panel of different restriction factors. We found that both human A3G and human tetherin can restrict XMRV. In contrast, XMRV does not appear to be sensitive to human TRIM5α restriction. Murine restriction factors can also inhibit XMRV. The virus is partially sensitive to mouse A3 and is inhibited by murine tetherin. Interestingly, it is restricted by both n- and b- alleles of Fv1.

Results

Development of a Rapid Assay for XMRV Using a Replication-Incompetent XMRV Clone. By cotransfecting a plasmid encoding XMRV cDNA (VP62, a gift of Robert Silverman, Lerner Research Institute, Cleveland Clinic) with an MLV-based vector encoding GFP (pCNCG) or LacZ (LTR-LacZ), it was possible to
produce reporter gene expressing XMRV for use in single-cycle infectivity assays, also reported in ref. 26. However, to improve safety as well as to increase packaging of the reporter construct, we synthesized a modified VP62 plasmid called HG1. Using site-directed mutagenesis, we removed sequences corresponding to both the packaging signal (nucleotides 293–388, as numbered in GenBank EF185282) and the U3 region (nucleotides 7720–8108) of the viral genome. Removal of either region should result in a replication incompetent clone: In this plasmid, expression of the viral RNA is under the control of the CMV promoter but, once reverse transcribed, the resultant cDNA will not encode a promoter. Removal of the packaging signal will significantly reduce the amount of XMRV RNA genome that is encapsidated in viral particles and will correspondingly dramatically increase the packaging of the cotransfected reporter vector RNA. Experiments using HG1 were performed at containment level 2.

**Differential Susceptibility of Cell Lines to XMRV Is Determined at a Postentry Level.** It has been shown that XMRV uses the xenotropic and polytropic MLV receptor, XPR1 (27, 28). This receptor is expressed on cells from a wide range of species, although polymorphisms in the gene in laboratory mice create a nonfunctional version of the receptor (28–30). To confirm that HG1 is functional and to test the infectivity of different commonly used cell lines in the laboratory, and thereby identify susceptible cell lines with which to study XMRV infectivity, we produced LacZ-encoding XMRV by cotransfection of 293T cells with the plasmids HG1 and LTR-LacZ, and challenged a panel of cell lines from different species. Productive infection was measured by the expression of β-galactosidase 48 h after infection. As shown in Fig. 1, the dog cell line D17, the cat cell line CrFK, two human cell lines, 293T and LNCaP, and the wild-mouse cell line *Mus dunni* all supported substantial XMRV infection in this single-cycle infectivity assay, while infection was ∼25- to 500-fold lower in the remaining lines: two laboratory mouse lines NIH (N)-3T3 and Balb (B)-3T3, HeLa cells, and three human T-cell lines, CEM, CEM-SS, and SupT1. The hamster cell line, CHO, did not support even background levels of XMRV infection. This was not because of expression of the reporter gene in these lines, as pseudotyped Mo-MLV particles expressing the same construct were equally infectious in HeLa and CHO cells as CrFK, 293T, and *M. dunni* cells (Fig. S1). To test whether a nonfunctional XPR1 receptor was responsible for the lack of infection in these cell lines, LacZ-encoding XMRV particles were pseudotyped with the G protein of vesicular stomatitis virus (VSV-G) and used to challenge the same panel of cell lines. For every cell line, the expression of VSV-G on XMRV particles mildly enhanced infection (Fig. 1). However, it did not fully restore viral infectivity in any of the poorly infectious cell lines to a high level, indicating that additional factors other than the envelope inhibit early stages of infection in these cells, and suggesting that XMRV could be a target for restriction factors.

**XMRV Is Inhibited by Exogenous Human and Mouse APOBEC Proteins.** Previously, it has been shown that Mo-MLV is strongly inhibited by overexpression of human A3G, but only weakly restricted by equivalent levels of the mouse homolog (mA3) (16, 19, 31–35). It is unclear how Mo-MLV is able to resist the antiviral effects of mA3, as it does not appear to express an equivalent of the Vif protein of HIV that induces hA3G and hA3F degradation. Although controversial, mA3 seems to be packaged into MLV particles and, when encapsidated into HIV particles at similar levels, it is able to effectively inhibit this virus (32, 34). Intriguingly, it has been reported recently that XMRV is able to replicate in human PBMCs (5), and it is well documented that these cells express A3G (7–9, 36). Therefore, we were interested to see if XMRV was resistant to hA3G in a similar manner to the resistance of Mo-MLV to mA3. LacZ-encoding XMRV or Mo-MLV were synthesized in the presence of various HA-tagged human APOBEC proteins or mA3 in 293T cells and equal titers of virus, as determined by RT-ELISA, were used to infect D17 cells. After 48 h, β-galactosidase activity was detected as a measure of infectivity (Fig. 2A). In
these single-cycle assays that monitor the early steps of viral replication, human A3G was able to inhibit the infection of XMRV to the same extent as Mo-MLV, more than 200-fold compared with the no-APOBEC control under these conditions. hA3B was also able to inhibit both viruses, but to a lesser extent, only 65- to 80-fold. All other human APOBEC proteins tested, hA3A, hA3C, hA3F (Fig. 2A), and hA3H (Fig. S2) reduced the infectivity of both viruses by less than 10-fold, despite being overexpressed at similar levels to those proteins that restricted infection (Fig. 2B). This result suggests that XMRV is susceptible to restriction by hA3G and hA3B. Although hA3B is poorly expressed in PBMCs (36, 37), hA3G is constitutively expressed, which raises the question: how can the virus replicate efficiently in the presence of exogenous mA3, although both these viruses were inhibited to similar extents by hA3G.

**XMRV Is Sensitive to Human and Monkey Tetherins.** In addition to A3G and A3F, human PBMCs express tetherin (also known as BST-2 or CD317), an IFN-induced membrane protein that restricts retroviral particle release by cross-linking nascent virions to the cell surface (14, 15, 38). Primate immunodeficiency viruses can counteract its action through either their accessory proteins Vpu (HIV-1) (14, 15), Nef (various SIVs) (39, 40), or envelope glycoprotein (HIV-2 and SIVgagTAN) (41, 42). No tetherin countermeasure has thus far been described for a gammaretrovirus, but because XMRV has been found to potentially infect tetherin-positive cells, we examined whether this virus could antagonize the antiviral action of various tetherin proteins. 293T cells were transfected with XMRV provirus in combination with a GFP-encoding retroviral vector (pCNCG) and increasing amounts of expression vectors encoding human, Rhesus, and African Green Monkey tetherins, and the resulting supernatants used to infect fresh 293T cells. Increasing expression of all of the tetherins tested lead to a profound decrease in released XMRV titer (Fig. 3A). Furthermore, in the case of human tetherin, the restriction of XMRV production could be almost completely reversed by expression of HIV-1 Vpu in trans (Fig. 3A, open squares). Thus, in transient-virus production assays, XMRV was highly sensitive to human and monkey tetherin expression, suggesting that it cannot counteract this restriction factor. However, in the case of HIV-2, transient 293T-based tetherin restriction assays do not accurately predict whether the virus encodes a tetherin countermeasure (14). We therefore went on to test whether XMRV could counteract tetherin in cells that constitutively express the restriction factor. HeLa cells released much lower titers of XMRV than 293T cells. We showed that this was in part caused by tetherin by the fact that expressing Vpu in trans increased XMRV release greater than 5-fold (Fig. 3B). This level of rescue was comparable to the 10- to 20-fold difference between wild-type and Vpu-defective HIV-1 in these cells (Fig. 3C). We reasoned that the lack of accessory genes in gammaretroviruses, and recent data from other retroviruses, suggested that if XMRV could counter tetherin activity, this attribute would be associated with its envelope glycoprotein (41, 42). Despite making a functional Env protein that pseudotypes retroviral particles (Fig. S3), expression of XMRV Env in trans in HeLa failed to rescue Vpudefective HIV-1 release to wild-type level (Fig. 3C). Finally, we could also show that XMRV was restricted similarly by murine tetherin expressed in human cells (Fig. S4), indicating widespread XMRV sensitivity to mammalian tetherins. Taken together, these data demonstrate that wild-type XMRV does not encode a human, primate, or murine tetherin antagonist and is, thus, highly sensitive to these restriction factors.

**XMRV Is Restricted by Fv1α and Fv1b but Not by Human TRIM5α.** The studies with APOBEC and tetherin proteins have shown that human restriction factors are capable of inhibiting XMRV. Moreover, experiments with mA3 have identified differences between XMRV and the prototype Mo-MLV. Therefore, we investigated the effects of a third class of restriction factor that targets the capsid protein of retroviruses. This group includes the murine protein Fv1 and the ubiquitous TRIM5α family of proteins (7, 12). Susceptibility of ecotropic MLV to these restriction factors is determined by specific residues in the viral capsid protein, notably residue 110 (23, 43, 44). Analysis of the capsid sequence of XMRV indicated that it most closely resembled B-tropic MLV and also encoded a glucatike acid at amino acid position 110 (Fig. 4A). Therefore, one might suppose that it had an identical restriction pattern to B-tropic MLV. GFP-encoding replication-incompetent XMRV, N-tropic MLV, or B-tropic MLV were produced by transfection of 293T cells and used to challenge cat CrFK cells that had been transduced with a plasmid expressing Fv1α, Fv1β, or one of a panel of TRIM5α proteins from different primate species and YFP from an internal ribosome entry site. The number of GFP- and YFP-expressing cells was measured by flow cytometry (Fig. 4B), and restriction was calculated by comparing the percentage of YFP- (restriction factor) positive cells that were also GFP- (virus) positive with the percentage of YFP-negative cells that were GFP-positive, and presenting this as a ratio (Fig. 4C). As expected, XMRV was restricted by Fv1β (Fig. 4 B and C). Only 5% of Fv1α-expressing
7% of Fv1b-expressing cells were infected, compared to 42% of 0.15. However, unexpectedly, XMRV was also restricted by Fv1b;
determinant of restriction speci
dentical and that both encode a glutamic acid at the primary
Consideration that the capsid sequences from each virus are 95%
difference between XMRV and B-tropic MLV is surprising,
over, it suggests that further residues may be important for CA-
pressed Fv1b; only 10.3% of Fv1b-expressing cells were infected,
observed, B-tropic MLV was very weakly restricted by overex-
control cells, a ratio of 0.18 (Fig. 4

taken to represent partial restriction.

Ratios of infected restriction factor-positive cells to restric-
tion (not shaded). Numbers between 0.3 and 0.7 (shaded gray) are
restriction (shaded black); ratios greater than 0.7 represent absence of
caption" and "restriction factor-negative cells. Ratios that are less than 0.3 are taken to represent
restriction (shaded black); ratios greater than 0.7 represent absence of (not shaded). Numbers between 0.3 and 0.7 (shaded gray) are
taken to represent partial restriction.

Discussion

whether a virus can infect particular cells within a specific host and the severity of the disease this may cause is dependent upon the many interactions that occur between viral and cellular factors. The initial interaction between retrovirus and host cell occurs between the viral envelope protein and the host cell receptor. In recent years however, there has been great interest in a group of proteins known as restriction factors (7). These cellular factors are thought to influence host susceptibility to infection, zoonotic transmission, and pathogenicity of retroviruses, and may represent previously unexplored targets for antiviral therapy. Thus, the effect of such factors on the replication of XMRV, recently identified as linked to two important human diseases—prostate cancer (1, 3) and CFS (5)—is potentially significant.

Although the expression of the XPR1 receptor is an essential requirement for XMRV infection (27, 28), by pseudotyping XMRV with the G protein from VSV, we have shown that differential susceptibility to XMRV in several cell lines does not track with receptor use or promoter activity (Fig. 1), implying that other cellular factors influence XMRV tropism. We therefore went on to analyze the effects of several human restriction factors on XMRV infectivity, to understand which of these factors may be important in determining host cell susceptibility in humans. Surprisingly, although XMRV has reportedly been isolated from human PBMCs (5), we show here that two human proteins known to be expressed in these cells can restrict XMRV infection: hA3G (Fig. 2) and tetherin (Fig. 3). This finding naturally raises the question of how this virus can replicate in human PBMCs. Further work is needed to truly define the cellular populations that harbor the virus in vivo, but there are various possible explanations for this apparent discord. The most obvious conjecture is that XMRV somehow evades these restriction factors in a natural infection. There are three scenarios for this:

First, it is possible that the levels of hA3G and tetherin are too low to inhibit XMRV infection in vivo. We have mainly examined the effects of exogenous proteins in our study. However, both hA3G and tetherin are expressed constitutively and broadly in hematopoietic cells (36, 46, 47) at levels that restrict HIV-1 infection. This would imply that significantly higher levels of these proteins are required to overcome XMRV compared to other retroviruses. Furthermore, the expression of tetherin and hA3G has been shown to be IFN inducible, at least in a subset of PBMCs (14, 36, 46, 47), and XMRV replication is reduced in response to IFN (27). In addition, CFS patients have been reported to have high levels of immune activation (5). It has been shown that expression levels of hA3G vary between individuals (36) and one A3G polymorphism, H186R, is associated with AIDS progression and declining CD4 T cells in HIV-1-infected individuals, although the in vitro antiviral activity of the two alleles was the same (48). Unfortunately, there is little data on expression levels of these proteins in prostate tissue, but a recent report suggests that apparent expression of hA3G in a panel of different tissues reflects the lymphocyte content of the sample rather than the tissue directly (36).

The second possibility is that XMRV infects a specific subpopulation of lymphocytes that do not express these restriction factors. Both hA3G and tetherin are constitutively expressed in activated T cells, B cells, dendritic cells, and macrophages (14, 36, 46, 47). However, the expression levels differ between these cell-

types, as does the level of induction seen in response to IFN. Indeed, the major target of XMRV may not, in fact, be a lymphatic cell but a different cell type altogether. Investigations into prostate cancer imply that XMRV can infect prostate tissue (1, 3). However, the studies disagreed as to what extent the tissue was infected and which cells within the tissue expressed viral proteins.

The third hypothesis is that instead of evading these restriction factors, XMRV has specific countermeasures to overcome hA3G and tetherin. It is worth highlighting that HIV-1 appears to have evolved two proteins, Vif and Vpu, specifically to overcome hA3 and tetherin proteins, respectively, in PBMCs. Clearly, there are no obvious candidates for such proteins in XMRV and no evidence of such activity in our experiments. However, it is possible that the virus could possess a countermeasure that is only effective in its native host cells. Identifying the in vivo target cells for XMRV will be critical for understanding its role in disease and its mode of transmission. Only when armed with this knowledge will we be able to prove the link between infection and symptoms and design appropriate therapies.

Alternatively, restriction factors may have an important role in limiting pathogenicity in vivo by partially inhibiting XMRV replication. Work in mice suggests that mA3 provides a degree of protection to mice against both mouse mammary tumor virus and MLV infection (49). Knockout mice bred on various backgrounds and that lack a functional A3 gene were more susceptible to infection with mouse mammary tumor virus (50), Friend-MLV (51, 52), or Mo-MLV (53). In fact, mA3 appears to map to the recovery from the Friend virus 3 (Rf3) susceptibility locus identified in the 1970s that protects mice from Fr-MLV through the production of a high-level antibody response and reduction of virus-induced erythropoiesis (51, 52, 54). Furthermore, the need for tetherin antagonism may be mitigated by cell-to-cell transmission in vivo. Thus, it may actually be beneficial for XMRV to show some susceptibility to human restriction factors. A low level of replication would be consistent with the near genetic identity of isolates from different individuals (6), although not necessarily with the apparent ease of isolation of particles from patient serum (5).

We have also shown here that XMRV is sensitive to restriction factors from nonhuman species (Figs. 2–4). To date, the origin of XMRV is unknown, but its close homology with murine endogenous MLVs makes mice the most likely source. Although endogenous MLVs are universally present, the homolog of polymorphisms in the XPR1 receptor (28–30), they must have infected the mouse at some point in history; therefore, it was interesting to observe that XMRV was sensitive to four murine restriction factors, mA3 (Fig. 2), Fv1a and Fv1b (Fig. 4), and murine tetherin (Fig. S4). Although the same arguments as above can be made to explain how XMRV could avoid these restriction factors in the mouse, these results may be informative for the study of restriction factors in general. There has been some disagreement in the literature as to the susceptibility of Mo-MLV to mA3 in vitro. Several groups have reported that MLV is unaffected by mA3 (19, 31, 33); others see a modest reduction in infectivity, as shown here (Fig. 2) (16, 32, 34). The resistance to mA3 has been attributed to exclusion of mA3 from MLV particles because of weak interactions between the NC protein and mA3 (33) or degradation of mA3 by the MLV protease (31). However, thorough investigations comparing the effects of mA3 and hA3G on MLV and vif-deficient HIV-1 have shown that mA3 is efficiently packaged into MLV particles, and so this explanation cannot readily account for the resistance seen (32, 34). Thus, the question remains as to how Mo-MLV protects itself from mA3 in vitro. It may therefore be revealing to use XMRV as a comparison with Mo-MLV to study their differing sensitivities to mA3 in vitro.

The susceptibility to Fv1 is also intriguing. Most wild mice have an intact Fv1 reading frame apart from Mus cooikii (55). However, some Fv1s from wild mice do not restrict N- or B-tropic MLV, which could easily explain why XMRV has not evolved to avoid restriction. Nevertheless, to our knowledge, this is a unique example of an MLV that is fully susceptible to both n- and b-alleles of Fv1, and as such, presents an opportunity to study the CA polymorphisms that are responsible for such restriction, particularly as XMRV is conversely resistant to all of the TRIM5 proteins tested (Fig. 4).

Finally, it is worth reiterating that XMRV has a different restriction pattern from the other MLVs tested here, and in particular from B-tropic MLV. Hence, it is not possible to predict whether XMRV will be restricted by any given factor merely by examining its protein sequence. Consequently, careful consideration needs to be given to potential restriction when designing XMRV studies in both cell lines and animals. If the links with prostate cancer and CFS are corroborated in future studies, then there will be endeavors to create animal models of infection for these diseases. Restriction factors could present major barriers to the success of these model systems (56). Using a relatively small panel of restriction factors, we have shown inhibition of XMRV not just by human proteins but also by restriction factors from mice and two monkey species. These results have important implications for two of the most common animal models. Although laboratory mouse strains no longer have a functional XPR1 receptor, knock-in strains could be generated relatively easily by crossing these mice with wild-mouse strains. However, as restriction is dominant, regardless of whether the wild-mouse strain expressed a functional Fv1 gene or not, the F1 mice would most likely restrict XMRV infection via Fv1 and mA3. Given the familiarity of the research community with monkey models for HIV infection, these may also present a logical animal model for XMRV infection. From our investigations, it seems that restriction by TRIM5ax would not be a significant problem in most species (Fig. 4), although both Rhesus and African Green Monkey tetherin proteins inhibited XMRV (Fig. 3). Obviously, testing the susceptibility of XMRV to every restriction factor from multiple species is beyond the scope of this study. However, it is important to draw attention to the potential issues posed by restriction factors.

In summary, we report here that exogenous expression of at least two human restriction factors is able to inhibit XMRV, a virus recently linked to two important human diseases. This finding presents new questions as to which cells the virus replicates in vivo, and how it evades restriction by these factors in PBMCs, particularly in the absence of obvious viral countermeasures. Restriction must also be an important consideration in the design of model systems for XMRV infection. Future work will establish whether this virus really does cause disease in humans, but efforts are already underway to find antiviral therapies and treatments. Restriction factors can now be added to the arsenal of possible defenses.

Materials and Methods

Plasmid Constructs. All plasmids are described in the text or have been described before (see SI Materials and Methods for further details).

Viral Infections. Viruses were prepared by cotransfecting 293T cells with plasmids encoding viral proteins and a packaging vector with or without specified restriction factors, as appropriate. Viral supernatants were harvested, filtered, and used to challenge the indicated target cells. Infection was measured by LaC assay or flow cytometry for GFP-infected cells. See SI Materials and Methods for detailed restriction assay protocols.

Immunoblot Analysis. HA-tagged APOBEC proteins were detected in whole cell lysates from transfected 293T cells using standard immunoblotting techniques and Li-COR Odyssey imaging. See SI Materials and Methods for further details.

ACKNOWLEDGMENTS. We thank Robert Silverman for the VP62 XMRV clone, Michael Malim for the HA-tagged APOBEC expression plasmids, and Jonathan Stoye for Fv1- and TRIM5-expression plasmids and helpful discussions. This work was supported by the U.K. Medical Research Council (File reference number U117583870) and the Wellcome Trust. K.N.B. and S.J.D.N. are Wellcome Trust Career Development Fellows.


