AIDS Rev 2003;5:156-164

Post-entry Restriction of Retroviral Infections

Greg J. Towers[1] and Stephen P. Goff[2]

[1]Wohl Virion Center, Department of Immunology and Molecular Pathology, Windeyer Institute, University College London, London, UK. [2]Howard Hughes Medical Institute, Columbia University College of Physicians and Surgeons, Columbia University, New York, NY, USA

Abstract

Pathogenic retroviruses have driven the evolution of several dominant-acting mechanisms able to block infection and protect the host. These are exemplified by the mouse gene Fv1, which encodes a Gag-like protein able to protect against murine leukemia virus (MLV) infection. The block is saturable, occurs after reverse transcription and is directed against the viral capsid gene. Several other mammalian species are also able to block MLV infection with the same capsid specificity. A human gene with this activity has been named Ref1. Recently, primates have been shown to restrict a variety of retroviruses only very distantly related to MLVs through a gene named Lv1. Restricted viruses include MLV as well as lentiviruses such as human immunodeficiency viruses 1 and 2, simian immunodeficiency virus and equine infectious anemia virus. In each case the block to one retrovirus can be saturated by co-infection with a second restricted virus. The possible mechanisms of action, and evolutionary consequences of restriction, are reviewed.

Key words

Retrovirus. Lentivirus. Restriction. Fv1. Ref1. Lv1.

Introduction

Retroviruses are a large and diverse family of enveloped, single stranded RNA viruses[1]. Their success throughout evolution is illustrated by their ubiquitous nature and high contribution of endogenous retroviral sequences to vertebrate genomes –at least 8% in humans[2]. Retroviruses have adopted a unique system of transferring genetic information. They carry an RNA genome that is reverse transcribed into DNA and then inserted into the host genome. Retroviral infection can lead to a variety of pathologies, including many different kinds of tumors and lymphomas as well as immune deficiencies, central nervous system defects and anemias.

Retroviruses are obligate parasites and as such depend on a variety of host-cell processes for the completion of their life cycle. Retroviral tropism is determined primarily by whether components of a given retrovirus are able to exploit each of the host-cell factors required for replication. However, retroviral tropism can also be restricted by the presence of dominant-acting inhibitory activities, expressed by target cells, that prevent the efficient progression of particular steps in the viral life-cycle. Infection by pathogenic retroviruses appears to have driven the evolution of dominant host mechanisms that inhibit a number of replication steps. An example is the recently identified cellular protein APOBEC3G/CEM15, a cytosine deaminase that destroys incoming DNA of human

Correspondence to:
Greg Towers
Wohl Virion Center, Department of Immunology and Molecular Pathology,
Windeyer Institute, University College London,
46 Cleveland St., London W1T 4JF, UK

immunodeficiency virus type 1 (HIV-1). The HIV-1 Vif protein counteracts the inhibitory activity of CEM15[3]. Another is Fv4, a proviral *env* gene that protects against murine leukemia virus infection by binding and blocking the ecotropic receptor[4]. This review concerns saturable post-entry blocks mediated by cellular factors called restriction factors.

Fv1: Restriction of murine leukemia virus in mice

The first retroviral restriction factor (Fv1) was described as a factor encoded by a mouse gene responsible for the resistance of inbred strains of mice to leukemia caused by murine leukemia viruses (MLV)[5]. The two original alleles of Fv1 enabled the division of MLVs into groups depending on their tropism for two inbred strains of mice, NIH and Balb/c[6]. N-tropic viruses are able to cause leukemia in NIH mice, but not in Balb/c mice. B-tropic MLVs are those with the opposite phenotype, causing leukemia in Balb/c mice, but not NIH mice. Thus, Fv1-N restricts MLV-B and Fv1-B restricts MLV-N. The critical difference between N- and B-tropic viruses was shown to be at position 110 in the capsid gene, with an arginine specifying N tropism and a glutamate B tropism[7]. Notably, a change from N to B causes a charge change from positive to negative. Fv1 is co-dominant in that heterozygous cells from a NIH x Balb/c cross are able to restrict both N- and B-tropic MLV[8], and mixed virus containing both N and B Gag proteins is restricted by either Fv1-N or Fv1-B proteins[9].

The most well-known and widely used MLV is Moloney MLV[10]. Moloney MLV is able to replicate well in both N and B cells, and causes leukemia readily in both N and B mouse strains, without any apparent sensitivity to Fv1. This virus is termed NB-tropic. Moloney MLV was extensively passaged through inbred mice and cell lines before infectious molecular clones were isolated[10,11]. Whether Moloney-like NB-tropic strains are commonly found in the wild is unclear; most MLV strains are sensitive to one or the other of the Fv1 alleles and it is possible that Moloney MLV may be a lab-adapted variant. The changes from N or B that make a virus NB-tropic remain undefined.

Much work, over many years, focused on the characterization of Fv1-mediated restriction. In cultures of cells from restricting mice, Fv1 was shown to cause up to a 1000-fold block of virus infectivity[12]. Restriction is saturable, and has been shown to result in two-hit kinetics of infection in some studies[13-15]. This means that the chances of a cell becoming infected are related to the square of the viral concentration, and the slope of a log (% infection) versus log (virus dose) is two. Put another way, if a stock of virus is diluted by 10-fold, and the number of infected cells drops by 10-fold, the kinetics are said to be "one-hit"; if the number of infected cells drops by 100-fold, the kinetics are said to be "two-hit". This non-linear relationship is due to the restriction factor being saturated and partially inactivated at high virus doses, allowing proportionally more cells to be infected at high dose than at low dose. For Fv1, multiple-hit kinetics are seen at high doses, and at low doses the slope shifts to one-hit, producing a characteristic bend in the titration curve. The presence of both one- and two-hit regions in the curve may explain why Fv1 restriction has been described to result in both single- and two-hit kinetics in different studies[16].

The fact that restriction is saturable is also demonstrated in abrogation experiments. In this case, pre-exposing cells to a high dose of restricted viral particles saturates restriction, and a second dose of sensitive virus is not blocked[17-19]. In other words, pre-exposure of target cells to restricted virions allows a second infection to proceed unrestricted. It is explained by the first dose entering the cells and saturating the available restriction factor, allowing virus in the second infection to proceed unimpeded. Abrogation is also seen if the two viruses are added at the same time. Abrogation is not observed with pre-exposure of cells to unrestricted virus, indicating that unrestricted virus cannot saturate the restriction factor, probably because it cannot bind to it.

When viral DNA synthesis is compared after Fv1-restricted versus unrestricted infection, the levels of linear viral DNA are similar[20,21]. However the formation of circular viral DNA, thought to be indicative of viral nuclear entry, is blocked in restricted infection. Taken together, these data are interpreted as showing that Fv1 acts after reverse transcription, but before nuclear entry. However, the fact that a polymerase minus mutant can titrate out a restriction factor in an abrogation experiment suggests that the interaction with the restriction factor can take place without DNA synthesis[22]. This is further discussed below. Certainly DNA synthesis is not required for interaction with Fv1. Virus can be restricted whether it enters cells via amphotropic envelope, ecotropic envelope or by the vesicular stomatitis envelope protein VSV-G[23,24], which are thought to utilize different routes of entry.

The Fv1 gene was cloned in 1996 and found to exhibit sequence similarity to a *gag* gene, related to endogenous retroviral elements (ERV-L) in mice and humans[25,26]. It appears that Fv1 is homologous to *gag* over a region extending from matrix through capsid and into the first part of nucleocapsid. The similarity to ERV-L of approximately 60% only extends to the Fv1 open reading frame (ORF). We presume that the Fv1 ORF has survived the loss of the surrounding retrovirus and is retained as a consequence of selective pressure provided by pathogenic MLV infection. Fv1 is expressed at very low levels and forms insoluble aggregates when over-expressed in mammalian cells or in bacteria (GJT unpublished data). These properties have made *in vitro* studies difficult and the mechanism of Fv1 restriction remains unclear. A mechanism depending on direct interaction

between incoming capsid and Fv1 would be consistent with a capsid determinant and saturable restriction, but such an interaction has not been demonstrated despite considerable efforts using the yeast two-hybrid and biochemical techniques. It is possible that a third cellular protein is required for the interaction.

The identification of the Fv1 gene allowed the development of an assay for restriction based on overexpression of Fv1 in permissive cells[27]. Fv1 is expressed together with a fluorescent protein, such as yellow fluorescent protein (YFP), and then cells are challenged with virus expressing a distinguishable marker, such as green fluorescent protein (GFP). After 48 h, expression of both proteins is measured simultaneously by two-color fluorescent activated cell sorting (FACS). This allows us to test whether cells expressing Fv1, the yellow cells, are protected from infection, i.e. from becoming green.

Overexpression of Fv1-B in Fv1-negative mink cells causes restriction of N-tropic virus as expected, but also weak restriction of B- and NB-tropic virus. This result suggests a broader inhibitory potential for Fv1-B than previously thought. Overexpression of Fv1 in cells naturally expressing Fv1 also produced interesting results. Overexpression of the endogenous protein (i.e. Fv1-N in NIH3T3 cells) did not increase the magnitude of restriction to MLV-B, suggesting that, despite very low endogenous levels of expression, Fv1 is not limiting. This result might suggest the existence of **a** limiting cellular factor. Overexpression of a different protein (e.g. Fv1-B in NIH3T3 cells) caused the restriction phenotype to switch to that of the overexpressed allele; NIH3T3 cells expressing high levels of Fv1-B are now no longer able to restrict MLV-B, but restrict MLV-N by about two logs. This is unlikely to indicate a competition between the Fv1 proteins for virus binding, as abrogation assays described above show that MLV-B cannot saturate, or compete for, Fv1-B. This result may be explained by the fact that overexpression of Fv1 produces insoluble aggregates. In this case, the aggregates might include most of the endogenous Fv1 because it is expressed in much lower amounts. The unincluded endogenous Fv1 would not be sufficient for function; but the unincluded exogenous Fv1 would be sufficient for activity. The Fv1 in the aggregates would be unavailable for restriction and so the cells become permissive for MLV-B. This result might also suggest a cofactor, as the overexpressed Fv1 could compete for cofactor rendering the endogenous Fv1 inactive.

The two-color restriction assay has also allowed a detailed mutational analysis of Fv1[28]. The N- and B-alleles differ at three points, with two amino acid substitutions and a variant C-terminus[25]. Analysis of chimeric Fv1 molecules demonstrated that the determinants of Fv1 specificity are complex. Possibly the most interesting result showed that Fv1-B with an Fv1-N C-terminus, the so called BBN mutant, is able to strongly restrict MLVs -N, -B and -NB. An Fv1 protein consisting of two functional domains is also suggested by the mutational analysis. A better understanding of the mutant Fv1 activities may await the solving of the Fv1 structure.

Ref1: Restriction of MLV in mammals

Southern-blot analysis has demonstrated that DNA from non-murine mammalian species, including humans, do not contain sequences closely related to Fv1[25] (GJT unpublished data). The ability of the pan-tropic VSV-G envelope to efficiently pseudotype MLV allowed the functional investigation of restriction in a range of mammalian cell lines[23]. Remarkably, a number of cell lines restricted N-tropic virus in a saturable manner, suggesting the presence of a second mammalian restriction factor, named Ref1 in humans.

Table 1. *Characteristics of resistance to retroviruses in various mammals*

Species	Factor/gene	Sensitive Virus	Reference
Mouse	Fv1	MLV	5, 6, 25
Human	Ref1	MLV-N EIAV	23, 29, 30
Hamster, Cow, Pig, Bat,		MLV-N	23 and GJT unpublished
Rabbit		HIV-1	35, 37
Chimpanzee		MLV-N	GJT unpublished
African Green Monkey	Lv1	MLV-N HIV-1 HIV-2 SIVmac EIAV	30, 35, 37, 38, 39
Rhesus Macaque		HIV-1	35, 37, 38
Owl Monkey		HIV-1	35, 37, 38
Squirrel Monkey		SIVmac	30, 35, 38

The properties of the resistance to retrovirus infection manifested by various species are listed. The mouse gene Fv1 is the prototypical virus resistance gene[25]; Ref1 and Lv1 genes are known by the restriction phenotype of cells from human and African green monkeys; the others are unnamed. Ref1 and Lv1 may be allelic. All species restricting MLV-N have been shown to have specificity for amino acid 110 in MLV-N capsid[23]. The determinant for HIV-1 and SIVmac restriction is also in the CA region, specifically the CAp2 region[38]. In each case where multiple viruses are restricted, the restriction to one virus can be saturated by co-infection with another restricted virus[30]. These species thus may contain a common restriction factor or restriction complex containing a common factor capable of restricting multiple unrelated viruses.

Saturable restriction to N-tropic MLV has been described in cells from humans, hamsters, pigs, cows, bats and African green monkeys (AGM). The block to infection in humans and AGMs is before reverse transcription and, as such, the levels of viral DNA synthesis are very low after restricted infection. As for Fv1, the determinant for Ref1 restriction of MLV is at amino acid 110 in capsid with an arginine causing restriction and a glutamate allowing infection[23]. It appears that these species express a factor which is similar to Fv1 in its specificity, but slightly different in its timing. Although fundamental mechanistic differences have been suggested based on the timing, the block before DNA synthesis may simply indicate a more efficient activity rather than a specifically earlier interaction. This study also showed that viral particles containing both N- and B-Gags (i.e. restricted and unrestricted Gags) were themselves restricted, even if only a small amount of restricted Gag was present[23]. This implied that restriction of MLV-N in human cells was likely due to a dominant acting factor.

There is an alternative explanation for an inability of N-tropic viruses to infect cells if the N-tropic capsids are unable to interact with necessary cellular factors in the restricting cell lines. This might be a consequence of the overall capsid charge –N-tropic capsid contains positively-charged arginine as oppose to the negatively-charged glutamate in B-tropic capsid. To distinguish between the lack of an activity and the presence of a restriction factor, abrogation experiments were performed[29]. It was shown that exposure of human cells to a high dose of N-tropic virus could abrogate restriction to a second dose of N-tropic virus. This ability to saturate restriction is good evidence for the presence of a dominant factor, Ref1, and for the notion that human cells have all the necessary factors for N- and B-tropic virus infection, but that Ref1 restricts N-tropic infection. Saturation of restriction by abrogation also converts the kinetics of infection from two-hit, indicative of restriction, to one-hit, indicative of permissive infection[29].

Recently it has been shown that a retrovirus from horses, equine infectious anemia virus (EIAV), is also restricted in human cells, extending restriction in humans to a lentivirus very distantly related to MLV[30]. EIAV virus-like particles (VLP) are able to saturate restriction to an infectious EIAV vector, indicating the presence of a titratable factor. Remarkably, MLV-N is also able to saturate the restriction to EIAV in human cells, extending Ref1's activity to the lentivirus family. The recognition of a gamma retrovirus and a lentivirus by the same cellular factor is surprising, given the evolutionary distance between them, but could be explained by similar three-dimensional capsid structures, despite a lack of significant sequence similarity. The recognition of multiple unrelated retroviruses by restriction factors is discussed below.

Lv1: Restriction of lentiviruses in primates

Chimpanzees infected with SIVcpz are the likely original source of HIV-1, and sooty mangabeys infected with SIVsm are the likely source of HIV-2[31,32]. Despite the primate origins of HIV-1 and -2, it has long been known that several primate species are unable to support the replication of HIV-1. Cell lines and peripheral blood lymphocytes (PBL) derived from rhesus macaque are unable to support HIV-1 infection, but are very permissive for SIVmac. This is not due to receptor differences between monkeys and humans, because an SIV bearing the HIV-1 envelope, a so-called SHIV, is highly infectious for macaque PBL[33]. The block to HIV-1 in monkey cells is dependent on the HIV-1 *gag-pol* gene[34]. Recently, experimental techniques such as those used to investigate Fv1 and Ref1 restriction have been used to investigate the dominant factors able to block infection by lentiviruses. Using VSV-G pseudotypes circumvented problems of lentiviral receptor expression, as the VSV-G envelope from vesicular stomatitis virus uses a receptor widely expressed on mammalian cells, allowing a very broad host range. Studies of cells from a series of mammals, including a broad range of primates, identified a number of species non-permissive for VSV-G pseudotyped lentivirus infection[35,36]. Examples of species non-permissive for HIV-1 are owl monkey, rhesus macaque, African green monkey and rabbit. Several groups investigated whether this was due to dominant or recessive factors. Pre-exposure of cells to high doses of HIV-1 increased the titer of a second HIV-1 dose by as much as fifty times in owl monkey cells, twenty times in rhesus macaque cells and ten times in cells from AGMs[37-39]. Recently it has been shown that AGM cells are also able to restrict HIV-2 and the divergent lentivirus EIAV[30]. Furthermore, cells from squirrel monkey were able to block SIVmac, but not HIV-1, in a similarly saturable manner[38]. Together, these data strongly suggested the existence of a restriction factor able to block a broad range of retrovirus infection in primates. This factor was named Lv1[38].

Cells from rabbits have been shown as being particularly resistant to HIV-1, but permissive to SIVmac[35]. Restriction of HIV-1 in rabbits might be a particularly interesting example. The block is very strong, around three orders of magnitude, but only one order of magnitude is saturable[37]; that is, abrogation experiments showed that treatment with high-dose HIV-1 can only increase the titer of HIV-1 by around ten-fold. This result might suggest that, whilst rabbit cells express a factor capable of restricting HIV-1, they also lack an essential activity required for efficient HIV-1 infection. This could be required for any aspect of the early or incoming part of the life cycle, and is uniquely required for HIV-1 and not SIVmac or MLV. However, high levels of restriction factor in rabbit cells could also explain this result.

Further evidence for the existence of a dominant factor Lv1 was provided by cell-fusion experiments, where the ability of permissive vs non-permissive cell-cell fusions to restrict HIV-1 infection was tested[38]. The products of fusing permissive 293T cells and restrictive OMK cells were shown to block HIV-1 infection to the same degree as unfused OMK cells. These experiments were also performed in AGM cells, and provided further strong evidence for the presence of a dominant factor able to block infection of HIV-1 in monkeys[39]. Abrogation experiments were used to show that both a polymerase minus HIV-1 and a virus containing an inactive reverse transcriptase were able to abrogate restriction[37,38]. These data demonstrate that neither reverse transcription nor any of the polymerase gene products are required for the virus to bind and saturate intracellular Lv1. A protease-minus virus, however, was unable to abrogate restriction, suggesting that Gag cleavage and maturation is required for restriction factor binding[38]. This would predict that cells expressing the Gag precursor, i.e. infected cells, would still restrict infection. This is true at least for MLV infected mouse cells, which remain able to restrict even after infection with restricted virus[40]. The determinant for restriction of HIV-1 was further mapped to the retroviral capsid-p2 domain, using an SIVmac in which the capsid gene had been swapped with the capsid from HIV-1. This virus contained all SIVmac sequence except for the capsid-p2 domain, which was from HIV-1. This SHIV behaved like HIV-1 rather than SIVmac, and was restricted in cells from owl monkey and rhesus macaque[38,41]. Furthermore, this virus was able to saturate restriction to wild-type HIV-1 in an abrogation experiment in simian cells[30].

Restriction of multiple divergent retroviruses by a common factor

The ability of cells from African green monkeys to restrict both gamma retroviruses, MLV-N and lentiviruses HIV-1, HIV-2, SIVmac and EIAV, allowed the investigation of whether a common factor in AGM cells (first seen as Ref1-like factor restricting MLV[23]) is responsible for the broad range of viruses blocked in these cells[30]. Both blocks to MLV-N and lentivirus, HIV-1 and SIVmac had been shown to be manifested prior to reverse transcription, to be saturable and to be present in the AGM cell line CV1[37-39]. Remarkably, cross abrogation experiments showed that exposure of CV1 cells individually to all restricted viruses listed above, but not unrestricted MLV-B, could saturate restriction to MLV-N[30]. This result strongly implies that a common factor exists, able to recognize multiple divergent retroviruses. It should be noted that the existence of different restriction factor complexes that share a common component could also explain these data.

MLV-N is much less efficient at saturating restriction to lentivirus, HIV-1, -2 or SIVmac. The reason for this is unclear, but may be related to the different behavior of capsid proteins from lentivirus and gamma retroviruses after viral entry. MLV has been shown to retain the capsid protein in the pre-integration complex, whereas HIV-1 sheds capsid early after viral entry[42,43]. This difference may be expected to affect the ability of the capsid protein to saturate the restriction factor. Similar experiments were performed in human cells, in which Ref1 is able to restrict MLV-N as well as the equine lentivirus EIAV[30]. MLV-N is able to effectively saturate restriction to EIAV, and EIAV is able to efficiently saturate restriction to MLV-N. Therefore, in this case (in human cells) cross abrogation works well with MLV-N as both a restricted virus and a saturating virus. Collectively these data suggest that in each species tested there is a common mechanism capable of recognizing and restricting divergent retroviruses, and that this mechanism utilizes a common restriction factor or possibly cofactor. It is intriguing that there are several examples of cells in which very closely related retroviruses are differentially restricted (e.g. MLV-N and -B in mouse cells), and of cells in which very divergent retroviruses appear to be restricted by a common factor (e.g. MLV-N and EIAV in human or AGM cells). We imagine that this is due to significant similarities in three-dimensional conformation of capsid structures, despite unrelated primary nucleotide sequences. Certainly there are common features apparent in the solved capsid structures of Human T-cell lymphotropic virus (HTLV), HIV-1 and EIAV[44].

The mechanism of restriction: some clues

Despite the fact that restriction was first demonstrated over 30 years ago[5], and Fv1 was cloned seven years ago[25], we know very little about the mechanism of restriction. There are a number of significant clues, perhaps the most obvious being that Fv1 is almost an entire *gag* gene and that the Gag protein, specifically capsid, is the target for restriction in all saturable blocks so far defined[23,25,38,45]. The simplest model for restriction involves a direct interaction between the restriction factor and the capsid of the incoming viral particle that somehow leads to inactivation of the virus[46]. Detection of such an interaction has remained elusive, despite considerable efforts from several laboratories. It is possible that a third cellular protein forms an essential part of the restriction factor/capsid complex, but it may be that the biochemical search for interaction has been confounded by the difficulty of working with interactions between a cellular protein expressed at very low levels (Fv1)[24], and a large complex of many viral and cellular proteins resident in the post-entry viral core complex.

Another clue involves the major homology region (MHR) and Fv1 intracellular localization. All retroviral Gag proteins, including Fv1 but excluding spumavirus Gags, contain a short conserved sequence near the middle of the capsid, known as the major homology region[26]. This sequence is required for infectivity[47,48] and is also required for Fv1 function[28]. The exact role of the MHR remains unclear. Many viral MHR mutants form apparently-normal particles, but the mutant viruses are unable to initiate reverse transcription upon entry[49]. Fv1-MHR mutants do not form aggregates when expressed in mammalian cells[24]. It seems possible that the MHR is required for some fundamental aspect of Fv1 multimerisation, and that the inability of Fv1-MHR mutants to restrict is related to this property. A recent paper has shown that wild-type Fv1, but not inactive mutant Fv1, is localized to the trans golgi network (TGN)[24]. The TGN receives cargo from the endocytic pathway as part of the route taken by some internalized receptors on their way back to the plasma membrane. It seems possible that the interaction between the restriction factor and the virus takes place as the virus is taken through the TGN after receptor interaction, and that an ability of Fv1 to multimerise with itself (and its target) is required for its activity.

Advances in fluorescent microscopy have enabled the investigation of the early part of the HIV-1 life cycle in real-time in live cells[50]. This methodology has provided some evidence for the involvement of the microtubule network for HIV-1 intracellular trafficking. Furthermore, examination of capsid shedding indicates that HIV-1 might associate with microtubules before loss of the capsid structure[50]. This observation raises the possibility that restriction might work by interfering with virus-microtubule interactions. If HIV-1 utilizes microtubules, we might expect other unrelated retroviruses to do so in a similar way, suggesting a common step that could be targeted by a single restriction factor. In this regard, it may be noted that the timing and extent of loss of CA from the PIC may differ among retroviruses. Thus, while HIV-1 CA may be lost relatively early after infection, MLV CA may be lost only later. These biochemical observations are consistent with the apparent differences in the timing of the blocks between Lv1 and Fv1. However, it is possible that the apparent differences in composition of the PICs are not significant to the restriction itself. These hypotheses are all very speculative.

A final intriguing clue is provided by the work of Berthoux, et al.[51]. These workers examined the known viral-stimulation activity of arsenic trioxide, and showed that Ref1 restriction of MLV-N is overcome by the addition of arsenic trioxide to restricted infection in human cells. The titer of HIV-1 is also increased by a few fold in human cells by arsenic treatment, probably reflecting a weak, but reproducible, block to HIV-1 infection by Ref1. Arsenic does not appear to have any effect on Fv1 restriction in mouse cells or Lv1 restriction in primate cells, indicating a specificity for Ref1, or for a human target. The mechanism of this effect is unclear, but a study of this phenomenon and a search for the cellular target of the arsenic effect is likely to give valuable clues to the mechanism of restriction and perhaps the identity of Ref1.

In summary, little is certain regarding the mechanism of restriction. The cloning of further restriction-factor genes may provide more clues. It will be important to discover whether the Ref1 and Lv1 gene products are also Gag-related molecules.

Evolutionary significance of restriction

Cells from a number of mammalian species appear to express retrovirus-restricting activities, with broad and unique specificities[5,23,37-39]. These data, together with the vast evolutionary distances among the species concerned, suggest that the occurrence of retrovirus-restriction factors in mammals is a widespread and probably ancient phenomenon. It seems likely that pathogenic retrovirus epidemics are responsible for the maintenance of these activities, but the identities of the retroviruses responsible for providing these selection pressures are unclear. MLV infection of mice is common, and it is likely that leukemogenic MLV infection has provided selection pressure for the acquisition of Fv1 from endogenous retroviral sequence. Wild mice are generally not able to restrict MLVs with specificity similar to either of the inbred NIH or BALB mice[52]. They do, however, generally have Fv1 open reading frames that appear to have been selected during murine evolution[53]. It is possible that their Fv1 genes have been selected for restriction of MLV more relevant to the local viruses providing selection pressure.

Human cells express a factor, Ref1, which restricts both N-MLV and an unrelated lentivirus, EIAV[23,30]. Only a relatively limited sample of primate lentiviruses have been examined for restriction in human cells, so it is possible that Ref1 has limited the transmission of these and other retroviruses to humans. There are probably many influences in addition to restriction factors that determine cross-species transmission of retroviruses. Whilst humans do not strongly restrict HIV-1 or HIV-2 infection, primary human cells from different individuals vary in their ability to support the replication of HIV-1 *in vitro*[54-56]. It is not clear at what stages of the virus life cycle this variation is manifested, but polymorphism in a restriction factor might contribute to and, more importantly, influence patterns of HIV-1 and HIV-2 horizontal transmission as well as disease progression. Addressing the question of whether Lv1 or Ref1 have significantly limited the prevalence of retrovirus-induced disease in primates, including humans, awaits the isolation and characterization of these factors.

SIV infection is widespread in African primates with about 50% of adult AGMs being infected[57,58]. The fact that geographically separated AGM subspecies harbor distinct phylogenetic subgroups of SIVagm suggests that African primates and SIVs have coevolved for a considerable period of time[59-63]. Consistent with this, the major coreceptor gene for SIVagm, CCR5, contains an unexpectedly high frequency of non-synonymous nucleotide polymorphisms. These polymorphisms are an important determinant for SIVagm infection, indicating that SIVs have selected for change in the AGM genome[64]. It is perhaps not surprising, therefore, that AGMs have evolved a factor such as Lv1 able to protect them from infection.

Significant differences in the resistant phenotype conferred by Lv1 are apparent among closely related AGMs[30], and this likely reflects subtle differences in selection pressures applied. Lentiviruses seem to have imposed selective pressure that has driven the evolution of factors such as Lv1 and CCR5 that can affect virus replication or transmission. It is intriguing that SIV infections do not appear to be pathogenic in their natural hosts, including SIVagm in AGMs, SIVcpz in chimpanzees and SIVsm in sooty mangabeys[31,65,66]. This mutual tolerance between virus and host is presumably due to a long co-evolution. Perhaps surprisingly, recent data has suggested that asymptomatic infection in monkeys is more dependent on tolerance of viral protein expression than reduction of systemic viral replication through the immune system. High virus loads appear to be tolerated long term in naturally SIV infected AGMs or sooty mangabeys without causing disease[66,67]. However, it is possible that significantly lower virus loads in the lymph nodes of infected AGMs may indicate the existence of mechanisms to limit viral replication at the local level rather than the systemic[66], and these may operate more efficiently in disease resistant species. Clearly it is important to better understand how such adaptations occur.

Future directions

The identification of further restriction factor genes, such as those encoding Ref1 or Lv1, is likely to provide insight into the genesis and mechanisms of action of restriction factors. It remains to be seen whether these factors are encoded by natural cellular genes or derived from retroviral sequences like Fv1. Either is certainly possible, and currently available data can be construed to suggest either. Fv1 was identified using classical mouse genetics and positional cloning; DNAs overlapping known markers linked to Fv1 were scanned for ORFs, and these candidates were then tested for the appropriate antiviral activity. This method cannot easily be used to clone genes in primates. An attractive approach to cloning Ref1 and Lv1 is a negative-selection protocol recently used to clone a gene able to block MLV infection in Rat cells at a late step of viral gene expression[68]. This strategy utilizes cDNA libraries cloned into a retroviral vector. The vector is used to deliver a library from a restricting cell line to a non-restricting cell line. A toxic restricted virus is then used to kill the bulk of the virus-sensitive cells that do not express the restriction factor, leaving a virus-resistant population. Single cell clones are then isolated, some of which should express the restriction factor. Candidate genes can be rescued by PCR and tested for their ability to restrict[27].

Yeast two-hybrid approaches have been tried to clone restriction factors, but recent data has suggested that this type of technique may not be appropriate in this instance. Firstly, Fv1 has not been shown to interact directly with either full-length Gag or capsid in a two-hybrid assay despite significant efforts. Panels of unrestricting cells containing single human chromosomes[69,70] have been screened unsuccessfully in an effort to identify the Ref1-positive human chromosome. The reasons for this failure are unclear, but may be due to aberrant expression of human genes in a foreign host. Radiation hybrids[71] may be more successful if a panel containing a useful combination of restricted/unrestricted hybrids can be identified.

It will be important to further define the determinant for lentiviral restriction. Alignments of *gag* sequences from MLV and lentiviruses suggest that the MLV determinant CA110 lies close to the cyclophilin binding site of HIV-1 Gag[72]. It will be interesting to investigate the influence of cyclophilin binding on HIV-1 restriction. If the determinant for restriction can be defined, it may be possible to make an HIV-1 able to replicate in macaques. This would significantly improve macaque models for testing HIV-1 vaccines that currently use fusions between HIV and SIV (SHIVs). Most SHIVs only contain HIV-1 envelope sequence, the rest coming from SIV. The fact that epitopes in Gag are important for immune escape of vaccinated macaques make this SHIV model less than ideal[73].

Retroviruses can cause fatal disease upon transmission to a new species, as is the case with HIV-1 and HIV-2 in humans and SIVsm in macaques[31,32]. Better understanding of the mechanisms that lead to tolerance of viral infection is likely to lead to better understanding of effective ways to treat HIV infection. We presume that, eventually, the human population could become able to tolerate HIV infection in much the same way as primates are able to tolerate SIV infection, but this eventuality is not likely to be effective in the near term, and such selection would be expected to be extremely costly in terms of human life. With a cumulative total of HIV infections at around 60 million, nearly 1% of the human population, the need to understand factors controlling host-virus relationships, and find new ways to control the HIV-1 pandemic are more important than ever.

Acknowledgements

We thank Subarna Bhattacharyya and Andrew Yueh for sharing unpublished data, and Yasuhiro Takeuchi for helpful comments. GJT is funded by a research career development fellowship no. 064257 from the Wellcome Trust. SPG is supported by PHS grant R01 CA 30488 from the NCI. SPG is also an Investigator of the Howard Hughes Medical Institute.

References

1. Coffin J, Hughes S, Varmus H. Retroviruses. CSHL Press. 1997.
2. Lander E, Linton L, Birren B, et al. Initial sequencing and analysis of the human genome. Nature 2001;409:860-921.
3. Sheehy A, Gaddis N, Choi J, et al. Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein. Nature 2002;418:646-50.
4. Ikeda H, Sugimura H. Fv-4 resistance gene: a truncated endogenous murine leukemia virus with ecotropic interference properties. J Virol 1989;63:5405-12.
5. Lilly F. Fv2: identification and location of a second gene governing the spleen focus response to friend leukemia virus in mice. J National Cancer Inst 1970;45:163-9.
6. Steeves R, Lilly F. Interactions of host and viral genomes in mouse leukemia. Ann Rev Genet 1977;11:277-96.
7. Kozak C, Chakraborti A. Single amino acid changes in the murine leukemia virus capsid protein gene define the target of Fv1 resistance. Virology 1996;225:300-5.
8. Rowe W, Hartley J. Studies of genetic transmission of murine leukemia virus by AKR mice. II. Crosses with Fv1-b strains of mice. J Exp Med 1972;136:1286-301.
9. Rein A, Kashmiri S, Bassin R, et al. Phenotypic mixing between N- and B-tropic murine leukemia viruses: infectious particles with dual sensitivity to Fv1 restriction. Cell 1976;7:373-9.
10. Moloney J. Biological studies on a lymphoid-leukemia virus extracted from sarcoma 37. I. Origin and introductory investigations. J Natl Cancer Inst 1960;24:933-51.
11. Shoemaker C, Goff S, Gilboa E, et al. Structure of a cloned circular Moloney murine leukemia virus DNA molecule containing an inverted segment: implications for retrovirus integration. Proc Natl Acad Sci USA 1980;77:3932-6.
12. Hartley J, Rowe W, Huebner R. Host-range restrictions of murine leukemia viruses in mouse embryo cell cultures. J Virol 1970;5:221-5.
13. Decleve A, Niwa O, Gelmann E, et al. Replication kinetics of N- and B-tropic murine leukemia viruses on permissive and nonpermissive cells *in vitro*. Virology 1975;65:320-32.
14. Pincus T, Rowe W, Lilly F. 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. J Exp Med 1971;133:1234-41.
15. Tennant R, Otten J, Brown A, et al. Characterization of Fv1 host range strains of murine retroviruses by titration and p30 protein characteristics. Virology 1979;99:349-57.
16. Jolicoeur P, Baltimore D. Effect of the Fv1 locus on the titration of murine leukemia viruses. J Virol 1975;16:1593-8.
17. Bassin R, Duran-Troise G, Gerwin B, et al. Abrogation of Fv1-B restriction with murine leukemia viruses inactivated by heat or by gamma irradiation. J Virol 1978;26:306-15.
18. Duran-Troise G, Bassin R, Rein A, et al. Loss of Fv1 restriction in Balb/3T3 cells following infection with a single N-Tropic murine leukemia virus particle. Cell 1977;10:479-88.
19. Boone L, Innes C, Heitman C. Abrogation of Fv1 restriction by genome-deficient virions produced by a retrovirus packaging cell line. J Virol 1990;64:3376-81.
20. Jolicoeur P, Rassart E. Effect of Fv1 gene product on synthesis of linear and supercoiled viral DNA in cells infected with murine leukemia virus. J Virol 1980;33:183-95.
21. Yang W, Kiggans J, Yang D, et al. Synthesis and circularization of N- and B-tropic retroviral DNA Fv1 permissive and restrictive mouse cells. Proc Natl Acad Sci USA 1980;77:2994-8.
22. Bassin R, Gerwin B, Levin J, et al. Macromolecular requirements for abrogation of Fv1 restriction by murine leukemia viruses. J Virol 1980;35:287-97.
23. Towers G, Bock M, Martin S, et al. A conserved mechanism of retrovirus restriction in mammals. Proc Natl Acad Sci USA 2000;97:12295-9.
24. Yap M, Stoye J. Intracellular localisation of Fv1. Virology 2003;307:76-89.
25. Best S, Le Tissier P, Towers G, et al. Positional cloning of the mouse restriction gene Fv1. Nature 1996;382:826-9.
26. Benit L, De Parseval N, Casella J, et al. Cloning of a new murine retrovirus, MuERV-L with strong similarity to the human HERV-L element and with a gag coding sequence closely related to the Fv1 restriction gene. J Virol 1997;71:5652-7.
27. Bock M, Bishop K, Towers G, et al. Use of a transient assay for studying the genetic determinants of Fv1 restriction. J Virol 2000;74:7422-30.
28. Bishop K, Bock M, Towers G, et al. Identification of the regions of Fv1 necessary for murine leukemia virus restriction. J Virol 2001;75:5182-8.
29. Towers G, Collins M, Takeuchi Y. Abrogation of Ref1 restriction in human cells. J Virol 2000;76:2548-50.
30. Hatziioannou T, Cowan S, Goff S, et al. Restriction of multiple divergent retroviruses by Lv1 and Ref1. EMBO J 2003;22:1-10.
31. Gao F, Bailes E, Robertson D, et al. Origin of HIV-1 in the chimpanzee Pan troglodytes troglodytes. Nature 1999;397:436-41.
32. Gao F, Yue L, White A, et al. Human infection by genetically diverse SIVSM-related HIV-2 in West Africa. Nature 1992;358:495-9.
33. Shibata R, Kawamura M, Sakai H, et al. Generation of a chimeric human and simian immunodeficiency virus infectious to monkey peripheral blood mononuclear cells. J Virol 1991;65:3514-20.
34. Shibata R, Sakai H, Kawamura M, et al. Early replication block of human immunodeficiency virus type 1 in monkey cells. J Gen Virol 1995;76:2723-30.
35. Hofmann W, Schubert D, LaBonte J, et al. Species-specific, postentry barriers to primate immunodeficiency virus infection. J Virol 1999;73:10020-8.
36. Ikeda Y, Collins M, Radcliffe P, et al. Gene transfer efficiency in cells of different species by HIV and EIAV vectors. Gene Therapy 2002;9:932-8.
37. Besnier C, Takeuchi Y, Towers G. Restriction of lentivirus in monkeys. Proc Natl Acad Sci USA 2002;99:11920-5.
38. Cowan S, Hatziioannou T, Cunningham T, et al. Cellular inhibitors with Fv1-like activity restrict human and simian immunodeficiency virus tropism. Proc Natl Acad Sci USA 2002;99:11914-9.
39. Munk C, Brandt S, Lucero G, et al. A dominant block to HIV-1 replication at reverse transcription in simian cells. Proc Natl Acad Sci USA 2002;99:13843-8.
40. Duran-Troise G, Bassin R, Wallace B, et al. Balb/3T3 cells chronically infected with N-tropic murine leukemia virus continue to express Fv1-B restriction. Virology 1981;112:795-9.
41. Dorfman T, Gottlinger H. The HIV type 1 capsid p2 domain confers sensitivity to the cyclophilin-binding drug SDZ NIM 811. J Virol 1996;70:5751-7.
42. Fassati A, Goff S. Characterization of intracellular reverse transcription complexes of Moloney murine leukemia virus. J Virol 1999;73:8919-25.
43. Fassati A, Goff S. Characterization of intracellular reverse transcription complexes of HIV type 1. J Virol 2001;75:3626-35.
44. Jin Z, Jin L, Peterson D, et al. Model for lentivirus capsid core assembly based on crystal dimers of EIAV p26. J Mol Biol 1999;286:83-93.
45. Boone L, Glover P, Innes C, et al. Fv1-N and -B tropism-specific sequences in murine leukemia virus and related endogenous proviral genomes. J Virol 1988;62:2644-50.
46. Goff S. Operating under a Gag order: a block against incoming virus by the Fv1 gene. Cell 1996;86:691-3.
47. Mammano F, Ohagen A, Hoglund S, et al. Role of the major homology region of HIV type 1 in virion morphogenesis. J Virol 1994;68:4927-36.

48. Bowzard J, Wills J, Craven R. Second-site suppressors of Rous sarcoma virus CA mutations: evidence for interdomain interactions. J Virol 2001;75:6850-6.
49. Cairns T, Craven R. Viral DNA synthesis defects in assembly-competent Rous sarcoma virus CA mutants. J Virol 2001;75:242-50.
50. McDonald D, Vodicka M, Lucero G, et al. Visualization of the intracellular behavior of HIV in living cells. J Cell Biol 2002;159:441-52.
51. Berthoux L, Towers G, Gurer C, et al. As2O3 enhances retroviral reverse transcription and counteracts Ref1 antiviral activity. J Virol 2003 (in press).
52. Kozak C. Analysis of wild-derived mice for Fv1 and Fv2 murine leukemia virus restriction loci: a novel wild mouse Fv1 allele responsible for lack of host range restriction. J Virol 1985;55:281-5.
53. Qi C-F, Bonhomme F, Buckler-White A, et al. Molecular phylogeny of Fv1. Mammalian Genome 1998;9:1049-55.
54. Williams L, Cloyd M. Polymorphic human gene(s) determines differential susceptibility of CD4 lymphocytes to infection by certain HIV-1 isolates. Virology 1991;184:723-8.
55. Spira A, Ho D. Effect of different donor cells on HIV type 1 replication and selection *in vitro*. J Virol 1995;69:422-9.
56. Eisert V, Kreutz M, Becker K, et al. Analysis of cellular factors influencing the replication of HIV type 1 in human macrophages derived from blood of different healthy donors. Virology 2001;286:31-44.
57. Kanki P, Kurth R, Becker W, et al. Antibodies to simian T-lymphotropic retrovirus type III in African green monkeys and recognition of STLV-III viral proteins by AIDS and related sera. Lancet 1985;i:1330-2.
58. Ohta Y, Masuda T, Tsujimoto H, et al. Isolation of simian immunodeficiency virus from African green monkeys and seroepidemiologic survey of the virus in various non-human primates. Int J Cancer 1988;41:115-22.
59. Allan J, Short M, Taylor M, et al. Species-specific diversity among simian immunodeficiency viruses from African green monkeys. J Virol 1991;65:2816-28.
60. Johnson P, Fomsgaard A, Allan J, et al. Simian immunodeficiency viruses from African green monkeys display unusual genetic diversity. J Virol 1990;64:1086-92.
61. Fomsgaard A, Hirsch V, Allan J, et al. A highly divergent proviral DNA clone of SIV from a distinct species of African green monkey. Virology 1991;182:397-402.
62. Hirsch V, McGann C, Dapolito G, et al. Identification of a new subgroup of SIVagm in tantalus monkeys. Virology 1993;197:426-30.
63. Jin M, Hui H, Robertson D, et al. Mosaic genome structure of simian immunodeficiency virus from West African green monkeys. EMBO J 1994;13:2935-47.
64. Kuhmann S, Platt E, Kozak S, et al. Cooperation of multiple CCR5 coreceptors is required for infections by HIV type 1. J Virol 2000;74:7005-15.
65. Villinger F, Folks T, Lauro S, et al. Immunological and virological studies of natural SIV infection of disease-resistant non-human primates. Immunol Lett 1996;51:59-68.
66. Holzammer S, Holznagel E, Kaul A, et al. High virus loads in naturally and experimentally SIVagm-infected African green monkeys. Virology 2001;283:324-31.
67. Rey-Cuille M, Berthier J, Bomsel-Demontoy M, et al. Simian immunodeficiency virus replicates to high levels in sooty mangabeys without inducing disease. J Virol 1998; 72:3872-86.
68. Gao G, Guo X, Goff S. Inhibition of retroviral RNA production by ZAP a CCCH-type zinc finger protein. Science 2002; 297:1703-6.
69. Cuthbert A, Trott D, Ekong R, et al. Construction and characterization of a highly stable human: rodent monochromosomal hybrid panel for genetic complementation and genome mapping studies. Cytogenet Cell Genet 1995;71:68-76.
70. Koi M, Shimizu M, Morita H, et al. Construction of mouse A9 clones containing a single human chromosome tagged with neomycin-resistance gene via microcell fusion. Jpn J Cancer Res 1989;80:413-8.
71. Rasko J, Battini J, Kruglyak L, et al. Precise gene localization by phenotypic assay of radiation hybrid cells. Proc Natl Acad Sci USA 2000;97:7388-92.
72. Stoye J. An intracellular block to primate lentivirus replication. Proc Natl Acad Sci USA 2002;99:11549-51.
73. Barouch D, Kunstman J, Kuroda M, et al. Eventual AIDS vaccine failure in a rhesus monkey by viral escape from cytotoxic T lymphocytes. Nature 2002;415:335-9.