



Declassification of Rodents Exposed to Third-Generation HIV-Based Vectors into Class 1 Animals

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Abstract

According to national and international regulations, experiments involving large amount of HIV-1 are generally restricted to infrastructures with a Biosafety Level 3 (BSL-3) status. However, work with genetically modified viral vectors such as the replication-defective lentiviral vectors developed by Trono & colleagues (Zufferey, 1997) is usually performed in a Biosafety Level 2 (BSL-2) containment, unless the inserted gene sequence belongs to a higher risk group. According to current safety procedures, animals exposed to recombinant vectors are classified in the same risk group as the vectors. This paper evaluates the risk of shedding of replication competent recombinant lentiviral particles by rodents infected with the third-generation lentiviral vector system and reconsiders the containment level of those animals.

Introduction

The development of gene therapy for incurable diseases is hampered because several important procedures involving animals are not compatible with current biosafety regulations. For instance, it becomes crucial to use the opportunity offered by the most modern imaging technologies such as Magnetic Resonance Imaging (MRI) to follow the effects of a gene therapy protocol in a non-invasive way. Such techniques afford more reliable results at the expense of fewer animals and less suffering.

BSL-2 containment aims at the prevention of uncontrolled transmission of certain types of viruses, or virus-derived vectors. Such containment is required only if viral or vector particles are shed from the animals. Lentiviruses are being extensively used for *in vivo* gene delivery in rodents, because they are able to transduce efficiently both dividing and non-dividing cells (Naldini et al., 1996). With the development of lentiviral transduction systems with improved safeguards (Zufferey et al., 1997 & 1998), work with HIV-based vectors may be performed in a BSL-2 environment (Dorsch-Haesler, 2001; NIH, 2002; Stanford University, 2005).

The third generation of lentivirus vectors provides

multiple safeguards against the production of a replication competent lentivirus (RCL) (Dull et al., 1998). In the third-generation system, the 4 viral accessory proteins are removed from the genome, the genetic elements are split into 4 plasmids and the U3 LTR promoter activity is deleted. Production of RCL can only be the result of 4 unlikely events: recombination of 4 plasmids and reconstitution of the U3 LTR promoter activity. Since the probability of the generation of an RCL during vector production is excessively low, vector batches would be contaminated with low number of RCL particles if any (Escarpe et al., 2003). Rodents are dead-end hosts for such RCL: viruses could enter into cells, but not produce any progeny *in vivo*. Infectious virus production by cells from HIV-transgenic mice was documented only *ex vivo* under special conditions (Wang et al., 2003). RCL amplification in rodents is therefore highly unlikely.

A system incorporating all of these safeguards can be seen as safe and is usually classified as BSL-2. However, such a classification is still constraining and limiting with respect of many modern technologies such as imaging and stereotaxis. Therefore, guidelines for the declassification of animals exposed to lentivector to BSL-1 are highly desirable.

In Switzerland, declassification guidelines were first based purely on vector half-lives determined on *in vitro*. Declassification was allowed after a period sufficient for the decay of all the injected particles. Studies determining the half-life of VSV G-pseudotyped vectors particles incubated in culture medium (DMEM) at 37°C showed that the half-life of such viral vectors was around 24 hours (DePolo et al., 2000; Kafri, 2001; R. Zufferey, unpublished data). Accordingly, in animals, assuming that all the vector particles injected in experiments would remain extra-cellular (worst case scenario), their half-life in body fluids could not exceed the 24 hours observed *in vitro*. Thus, in the practice, declassification of an animal injected with 10^6 particles was allowed after 21 days, because it takes 20 days for the decay of 10^6 particles to one single biologically active particle ($2^{20}=1,048,376$). Because the shedding of biologically active vector or virus particles is the reason for BSL-2 containment, it was previously

decided that such containment was not justified for more than 21 days after the injection (Statement of the Swiss Expert Committee for Biosafety from June 30, 2004).

As mentioned above, such a long BSL-2 containment is too constraining. Therefore a revision of the recommendations based on experimental *in vitro* evidence became important. In this paper, we propose new recommendations based on the real *in vivo* particles' half-life determined experimentally. To this end, we have tested vector particles recovered from rat plasma after intravenous (IV) injection of 10^7 transducing units (TU). Based on our results, we propose that rodents infected intravenously with high titer of pHR-based lentiviral particles can be declassified, depending on the injection site, to BSL-1 much earlier than 21 days. We propose that the animal experiment can be declassified to BSL-1 at 72 hours post infection in animal injected intravenously and 24 hours post infection for animals injected directly into the brain.

Materials and Methods

Choice of a Suitable Cell Line

To determine the tolerance of different cell lines toward rat plasma, we have added different amounts of rat plasma (from healthy control animals) to 293T, HeLa and F208 cells. We tested 25, 50, 100, 200, and 400 μ l of rat plasma, with the volume of culture medium remaining constant at 1 ml. We observed that cells of human origin (293T and HeLa) reacted negatively to increasing dose of rat plasma (resulting in growth arrest to cell death) whereas the rat cells F208 were comparable to control up to 200 μ l of rat plasma.

Plasmids

Four plasmids were used to prepare the vector batch used for all the experiments described in this report: The transfer vector plasmid pHR'CMV-LacZ-WPRE has been described previously. It is identical to the pHR'-CMV-LacZ plasmid whose sequence is available under the Gene bank accession number AF 105229 (Naldini et al., 1996), except for the addition of the WPRE cassette (Zufferey et al., 1999) downstream of the LacZ open reading frame. The Rev encoding plasmid pRSVrev, the VSV G encoding plasmid pMD.G and the packaging plasmid pCMV Δ R8.92 (encoding HIV-1 Gag/Pol and Tat) have also been described previously (Dull et al., 1998).

Vector

The lentiviral HR'CMVLacZWPRES vector batch (Zufferey et al., 1999) used in all of the experiments had a titer of 10^9 TU/ml in HeLa cells with a p24 concentration of 102 μ g/ml. This vector batch has been prepared by transient transfection of 293T as described in Zufferey and Trono (2000). Concentration of the capsid protein (p24) is a measure of the number of vector

particles in a suspension. Usually, in our hands, 1 ng of p24 corresponds to 10^4 TU. Monitoring of p24 concentration can also be used for RCL detection (Escarpe et al., 2003). Prior to the injections, 10 μ l (10^7 TU) from the vector stock were diluted into 90 μ l PBS to prepare a 100 μ l suspension ready for use.

Vector Titration in Rat Plasma

Plasma was collected and 200 μ l were used for titration. Plasma samples were applied on 2×10^5 F208 cells in a total culture medium volume of 1ml. After 48 hours, Lac Z activity was revealed by histochemistry according to standard protocols.

As controls, a series of vector dilutions was prepared, in either PBS or plasma from normal rats, and used in parallel to transduce F208 cells. Transduction by the lentivector was not inhibited by the presence of rat plasma.

Tail Vein Injection

Two rats received 10^7 TU by vein tail injection and were sacrificed 24 hours after the injection. Plasma was collected and vector particles titrated.

Femoral Vein Injection and Vector Particles Recovery from Plasma

Two rats, about 300 g, were anesthetized and kept under general anesthesia for 2 hours using Halothane. The femoral vein was surgically exposed by a skin incision. A catheter (pediatric butterfly) was inserted into the femoral vein and kept in the vessel during the whole experiment. The 100 μ l vector suspension containing 10^7 TU was pushed through the catheter with another 100 μ l of PBS. The animals were sacrificed at the end of the experiment.

Blood was sampled at 10 minutes, 60 minutes, and 120 minutes after the injection. At each time point, 0.5 ml blood was taken by connecting a heparin-coated syringe to the catheter. Blood samples were transported on ice and centrifuged within 5 minutes. Cells and plasma were separated by 1 minute centrifugation at 13000 rpm. 200 μ l of plasma was gained and added onto F208 cells for titration.

Detection Threshold

200 μ l out of 10 ml of plasma are tested on F208 cells (2% or 1/50 of total plasma volume). The detection threshold is therefore 50 TU per animal (or 1 TU in 200 μ l plasma).

Results

After injection of 10^7 TU of LacZ lentivector into the tail vein of two adult rats, we were unable to detect the presence of any transducing units in 200 μ l of plasma 24 h post injection (data not shown). In control experiments, transduction of 208F cells by the same

lentivirus was; however, shown not to be inhibited by the presence of rat plasma.

The absence of TU 24 hours after the injection prompted us to look at earlier time points. To evaluate the *in vivo* half-life of vector particles in plasma, we decided to inject animals in the femoral vein using a catheter. Taking advantage of the catheter, blood samples (0.5 ml) were collected at 10, 60, and 120 minutes post-infection and analyzed for the presence of biologically active vector particles (Figure 1). As shown in the second part of the figure, our data indicates that 10 minutes after injection the load of vector particles is still very high. During the first hour, at least a 4 log decrease in the number of transducing particles can be observed. Finally, as early as 2 hours after injection, the vector load has been cleared from the blood of infected animals (Figure 1, panel B). The setting of the experiment, (general anesthesia of the animal for 2 hours plus 0.5 ml of blood taken at each time point) does not allow a longer experiment. However, based on these results, one can extrapolate that after an incubation period of 24 hours most, if not all, vector particles and potential RCL are removed from the circulating blood.

Discussion

A safety evaluation of viral vector systems has to take into account both the vector system design and the host organism.

A System with Several Built-in Safeguards

The development of safe lentiviral vectors requires: (i) the elimination from the system of any dispensable component such as gene encoding accessory proteins and promoter sequences in the LTR, (ii) the separation of the system components into as many plasmids as possible and (iii) limiting sequence overlaps, or homologies between the plasmids encoding the vector components. The so-called third generation lentiviral vector system fulfills most of these requirements: (i) the complete exclusion of 4 accessory proteins thought to be necessary for HIV pathogenesis from the packaging cells, (ii) the distribution of the components on 4 different plasmids with minimal overlap (split design) and (iii) the deletion of the promoter activity in the 3' LTR (self-inactivation).

These safeguards provide protection against the generation of RCL by different mechanisms. First, genetic information of three plasmids must be recombined in a precise manner to the fourth one in order to reconstitute a replication-competent genome. At least 3 unlikely recombination events are required for this. Second, the LTR must regain a promoter activity, which requires a fourth recombination event. Third, the 4 accessory proteins are irreversibly eliminated from the system; no recombination event can bring them back. The absence

of these accessory proteins has a major incidence on the replication fitness and pathogenic potential of a putative RCL as shown for SIV by Desrosiers et al. (1998). Finally, the absence of most of the HIV-1 envelope gene from the combined total all plasmid DNAs used ensures that HIV-1 will not be reconstituted provided a heterogenous *env* gene (not of retroviral origin) is used.

The occurrence of an RCL is an extremely rare event; the frequency of RCL appearance is much lower than the detection threshold of the most sensitive tests currently available (1 RCL in 10^8 transducing units) (Escarpe et al., 2003; Wu et al., 2000). In addition, the following observations have to be taken into account. The HIV-1 R8.91 molecular clone carrying the 400bp deletion in the LTRs was produced by transfection of 293 T cells. Infection of C8166 cells with this defective virus showed that the deletion in the 3'LTR is repaired in about one in every 50,000 vector's particles (R. Zufferey, unpublished data). A similar observation was also reported on the repair of the foamy virus LTRs (Bastone & Lochelt, 2004). Indeed, of all recombination events involved in RCL generation, LTR repair is the recombination event with the highest probability, because there is an extensive sequence identity between the 5' and the 3' LTRs (Bastone & Lochelt, 2004). Similarly, one could try to determine the frequency at which both the 5' and the 3' LTR are transferred from the vector plasmid to the packaging plasmid in a two-plasmid system. In principle, a probability could be experimentally assigned to each one of the 4 recombination events required for the generation of a RCL. Assuming that these recombination events are independent events, the probability of RCL generation is obtained by multiplying the probability of each single event. In a pessimistic estimation, one would give to all 4 events the same probability as for the most frequent one LTR repair (rounded up to 10^4). RCL would then be generated at an estimated frequency of 10^{-16} ($10^4 \times 10^4 \times 10^4 \times 10^4$). To put this number in context, it should be considered that a maximal vector dose for an intra-cerebral injection in rats is 10^7 TU. Therefore, only one rat out of a billion injected would receive one RCL.

Also important when considering the complementation of the deletions within the vector plasmid is the apparent absence of helper functions for lentiviruses in human cells that have not been previously infected with a lentivirus. In contrast to murine cells, which contain multiple endogenous sequences capable of complementing and/or recombining with MuLV-based vectors, this does not appear to be the case with human cells and lentiviral based vectors.

Non-Permissive Hosts

HIV-1 is a strictly species restricted virus, making the establishment of an animal model for AIDS very difficult. Not even in the primates the most closely related to

Figure 1

Half-life of vector particles in plasma. 10^7 TU were injected into two rats and at various time points plasma was collected. For each time point, 200 μ l of plasma (1/50 of total plasma volume containing potentially 2×10^5 RCL) were analyzed on F208 cells. A rapid decay in the number of vector particles, or potential RCL can be observed after 10, 60, and 120 minutes. (A) β -Galactosidase staining of HR'CMVLacZWPRE F208 positive cells. (B) Decrease in the number of vector particles expressed as a percentage of the total amount of injected lentivector.

Figure 1A

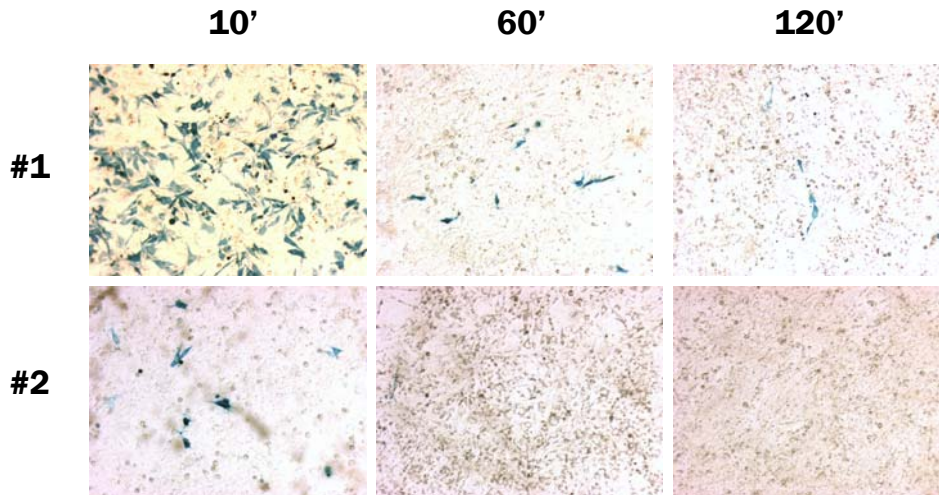
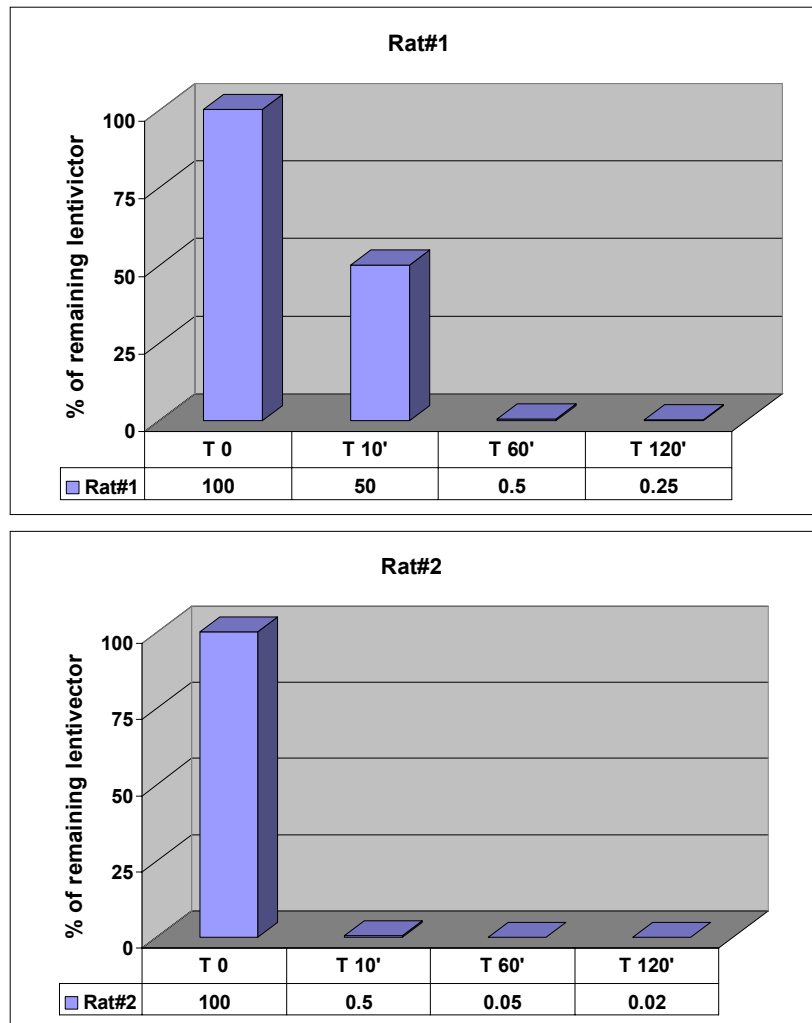


Figure 1B



humans does HIV replicate efficiently (Joag, 2000). The non permissivity of rodents for lentivirus replication is therefore a key point to consider in the biosafety evaluation of lentiviral vectors.

HIV-1 Replication in Rodents

In rodents, at least 3 major replication blocks have been identified and recently defined at the molecular level. The first block is at the cell entry step: HIV-1 envelope protein cannot use rodent CD4 and CCR5 to enter into rodent cells (Doranz et al., 1997). This block is of minor interest for the biosafety assessment, because the functional HIV-1 envelope gene is not present. However, two other major blocks prevent the replication of HIV after the entry step and cannot be circumvented by pseudotyping (endogenous or heterologous envelope protein provided in trans to a replication-defective vector that lacks the envelope coding sequence in order to alter the tropism): (i) transcription of HIV in rodent cells is inefficient. This is due to amino acid sequence differences between human and rodent cyclin-T1, the host co-factor for the HIV-1 trans-activator Tat (Taube et al., 1999; Garber et al., 1998); and (ii) viral transcripts are spliced excessively in rodent cells with a significant decrease in both the unspliced 9 kb transcript forming the viral genome and encoding Gag and Gag-Pol, and in the singly spliced transcripts encoding the Vif, Vpr, Vpu, and Env proteins (Trono & Baltimore, 1990; Malim et al., 1991; Bieniasz & Cullen, 2000). This excessive splicing is due to a lack of Rev-assisted nuclear export of viral messenger RNA (Bieniasz & Cullen, 2000). In addition, less well characterized blocks are likely to contribute to the non-permissive status of rodent cells for HIV-1 (Mariani et al., 2000). As a good illustration of mouse permissibility to HIV-1, one can cite all of the studies modeling HIV-induced encephalopathy by intra-cerebral injection of HIV-1-infected human macrophages in SCID mice. In all instances, immuno-reactivity to the p24 viral core protein disappears with the clearance of the human cells (Dou et al., 2003).

Replication in Rodents of Multiply Attenuated VSV-G Pseudotyped RCL

Transgenic mice and rats expressing human CD4 and human CCR5 as HIV-1 receptor and co-receptor provide the appropriate model to mimic the situation where there is no entry block (Keppler et al., 2002; Keppler et al., 2005). All other replication blocks remain active. Co-cultures of human Peripheral Blood Mononuclear Cells (PBMC) with splenocytes, or lymphocytes from CD4/CCR5 transgenic mice challenged with wild-type HIV-1 could never demonstrate the production of any replication-competent HIV-1 by murine cells (Browning et al., 1997).

Another study by Keppler et al. (2002) provided

further evidence for the non permissivity of rats. Rats transgenic for the same genes as above were challenged with 10^7 TCID₅₀ (Tissue Culture Infectious Dose 50) intra-peritoneal and analyzed for viral load (viral genome copies/ml plasma) weekly for 7 weeks. HIV-1 genome was never detected in plasma from non-transgenic rats. Among the transgenic rats, 1 never tested positive, 2 tested positive only once out of 7 time points and 3 tested positives on 2 time points. In the positive samples, viral load was less than 150 genome copies/ml. This study is remarkable for the extremely high dose injected: 10 times the highest vector dose for rats. In other words, when the entry block is circumvented, rodents are still not permissive to wild-type HIV-1. One can easily postulate that they would be even less permissive to four-time attenuated RCL.

Potential for Transmission from Animals to Humans

Lentiviral particle shedding after intra-cerebral injections requires that particles have reached the vascular compartment. Through blood flow, particles could reach body fluids such as saliva, urine, and feces. Only a minority of particles will access the vascular compartment. The risk of transmission of a replication-competent virus from rodent to human is therefore minimal.

Generation of New Hybrid Viruses is Unlikely

The only potential replication-competent viruses to be considered here are replication competent lentiviruses (RCL) generated during vector production. There are 2 reasons for that: (i) because rodents are not infected with any lentivirus, recombination between the HIV-1-derived sequence and another lentivirus can be excluded; and (ii) recombination of lentiviral sequence and endogenous murine retroviruses is not a concern because the phylogenic distance separating lentivirus from other retroviruses is too large to result in functional hybrids.

Conclusions

Several conclusions can be drawn from our data. First, the frequency of RCL in vector batches is lower than 2×10^{-5} [1/200 000], which corresponds to less than 50 RCL in 10^7 particles. Second, due to the rapid decay of the circulating virus, we assume that no vector or RCL shedding will occur three days after intravenous injection in rodents infected with a third-generation lentivirus vector system. Indeed, 24 hours after intravenous injection of 10^7 TU of lentiviral vectors, less than 50 TU were still circulating in our experiment. Based on these observations, we propose that animals infected with a third-generation lentiviral vector can be handled safely in a BSL-1 containment environment after an appropriate period of time has passed to ensure that in vivo

mechanisms have eliminated circulating viruses: 3 days after IV injection and 1 day after intra-cerebral (i.c.). Indeed, we propose that animals infected directly into the brain can be declassified 24 hours after injection since the proportion of particles that can pass into the vascular system after an i.c. injection is negligible. These recommendations apply only for rodent in vivo procedure, then only for non invasive procedures.

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