State-of-the-Art Lentiviral Vectors for Research Use: Risk Assessment and Biosafety Recommendations

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Abstract: Lentiviral vectors (LV) are competent gene transfer vehicles, as used for both research and gene therapy applications, because of their stable integration in non-dividing and dividing cells and long-term transgene expression. Along with our understanding that LV offer solutions for gene therapy, biosafety concerns have uncovered risks due to insertional mutagenesis, the generation of replication competent lentiviruses (RCL) and vector mobilization. Researchers therefore continue to devote significant efforts in designing LV with improved efficacy and biosafety features.

The choice of a particular LV system for experimental studies is often driven by functional considerations, including increased productivity and/or transduction efficiency. The design of safer vectors has also directly benefited researchers allowing them to conduct experimental studies with lower risk. Currently, vectors combine improved safety features (that decrease the risk of recombination and vector mobilization) with increased transduction efficiency. Hence, risks associated with the inadvertent transduction of cells of the investigator gain greater importance in assessing the overall risk of these vectors and become an important biosafety concern.

This review outlines the different strategies used to improve LV biosafety by comparing state-of-the-art and emerging LV production systems and highlighting biosafety issues that can arise during their contained use. The few existing national and international biosafety recommendations that specifically address the use of LV in research are discussed and recommendations for most common research activities using LV are proposed.

Keywords: Lentivirus, vector production, biosafety, vector design, guidelines, HIV-1 derived vector, gene transfer, split-packaging.

1. INTRODUCTION

Viruses are biological agents that efficiently introduce their genetic material in a target cell upon infection and depend on the host cell for their replication. Vectors harbour genes of interest in place of the wild-type viral genes from which they are derived. Hence, non replicating vectors lack the genetic information for self-propagation in cells but retain the capacity for introducing genes of interest into the target cells. Lentiviral vectors (LV) are derived from viruses belonging to the retrovirus family (*Retroviridae*, genus lentivirus). LV are currently one of the most practical gene transfer vehicles, both for research and gene therapy applications because of their stable integration in dividing as well as non-dividing cells and long-term transgene expression. Human immunodeficiency virus type I (HIV-1) is probably the best-studied lentivirus, and despite its well-known human pathogenicity, it rapidly became apparent that HIV-derived vectors offered unique gene therapy solutions for differentiated and non-dividing cells. Thus, considerable research has been devoted to designing LV with improved biosafety features. Furthermore, non-gene therapeutic applications of LV have also been widely explored, including gene function studies based on stable gene knock-down using RNA interference in mammalian cells. Although the use of LV in experimental studies is likely driven by their functional qualities, including increased productivity and/or transduction efficiency, the design of safer vectors has directly benefited scientists interested in improving the safety of their experiments.

The use of LV, which are considered as recombinant viruses, or the use of LV transduced cells may fall under the scope of several regulatory provisions and guidelines depending on the purpose or the type of activity involved. Relevant regulations, guidelines and advisory authorities that should be considered or consulted whenever using LV generally aim at: (i) protecting the workers from risks related to biological agent exposure in the workplace [1-3]; (ii) regulating the construction, handling or deliberate release of genetically modified (micro-) organisms or organisms containing recombinant DNA molecules [4-7]; and (iii) ensuring the safety of biopharmaceutical products and medicinal products

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for human or veterinary use, such as those taking benefit of LV or LV transduced cells [6, 8, 9].

Although the use of LV or lentiviral transduced cells for therapeutic purposes gives rise to a number of critical considerations including quality, efficacy, safety, ethical, social and regulatory issues, the scope of this review is limited to the biological risk assessment for LV in research activities. Our goal is to provide an overview of the different strategies currently employed for improving vector biosafety and compare state-of-the-art lentiviral packaging systems. While this review focuses on hazards posed by the vector alone, we do recognize that LV risk assessment should also take into account the hazards associated with the transgene. Cloning an oncogene into LV or enabling shRNA mediated knockdown of tumor suppressor genes clearly remain high risk manipulations requiring that stringent biosafety measures be implemented. This review will consider vector choice in relation to the host system and include a discussion of current biosafety recommendations specifically addressing LV use and the emergence of safer LV production systems.

2. POTENTIAL ADVERSE EFFECTS ATTRIBUTABLE TO LENTIVIRAL VECTORS

The majority of adverse effects observed with lentiviral vectors are actually common to all retroviral vectors because they are linked with the retrovirus life cycle, based on integrating the viral genome into the host cell genome. Upon infection these viruses reverse transcribe their single-stranded RNA genome into a double-stranded DNA intermediate, which is then integrated in the host genomic DNA thereby giving rise to the provirus. Proviruses can be inserted at a wide variety of sites in the host genome, with the Long Terminal Repeats (LTRs) flanking an internal virus coding region.

One of the main adverse events that must be considered is the potential generation and propagation of replication-competent lentiviruses (RCL) during vector production, something that always needs to be excluded during GMP-production for clinical trials. The generation of RCL is believed to occur through homologous recombination between overlapping sequences. Though replication competent retroviruses have been detected in split-function retroviral packaging cell lines using ecotropic or amphotropic retrovirus envelopes [10, 11], to date no RCL events have been reported for lentiviral packaging systems. This may partially depend on vector architecture since self-inactivating vectors (SIN) are less likely to produce RCLs (*see infra*).

Another event common to all retroviruses is the integration of viral DNA in the host genome with its associated risk of insertional mutagenesis and/or the transactivation of neighbouring genome sequences. In a number of cases, adverse events such as oncogenesis have occurred following retroviral integration. A good example is insertional activation of the LMO2 gene (a known proto-oncogene) by the LTR enhancer observed after introduction of gamma-retroviral vectors, which was found to contribute to leukaemogenesis in five patients treated by gene therapy for X-linked severe combined immunodeficiency in two separate clinical trials [12-14]. The pathogenesis of a murine acute myeloid leukaemia that followed retroviral vector gene transfer was also associated with insertional activation of a proto-oncogene in the vicinity of the retroviral insertion locus [15]. Another study found a high incidence of oncogenesis following gene transfer with EIAV (equine infectious anaemia virus)-derived lentiviral vectors in neonatal mice [16], although it was not clear whether the observed oncogenesis was associated with insertional mutagenesis or transactivation. Most recently, clonal dominance has been detected in one of the subjects of an ongoing clinical trial using heamatopoietic stem cells transduced by a self inactivating (SIN) HIV-1 lentiviral vector with chromatin insulators [17]. The clonal dominance appears to result from the integration of the vector in the gene encoding for the HMGA2 protein, which is associated with both benign and malignant tumors [18]. An increased risk for cancer development risk in HIV-seropositive individuals has also been observed and includes Kaposi sarcoma associated with HHV-8 infection [reviewed in 19] and Burkitt lymphoma associated with EBV infection [reviewed in 20], both resulting from reactivation of these adventitious viruses in patients with immunodeficiency, and in extremely rare cases, HIV-1 associated T-Cell lymphomas. In the latter case HIV-1 has been shown to be clonally integrated in the CD4+ T cell genome just upstream from the *c-fes* oncogene where the integrated HIV-LTR acted as an enhancer element for *c-fes*, leading to upregulated Fes expression [21-23].

Contrary to earlier thinking, integration of retroviruses in the host genome is not a purely random event. Studies have now shown that both gamma-retroviruses and lentiviruses preferentially integrate in transcriptionally active genes [24-29]. HIV-1 integration is influenced by a number of different factors including base composition [30], Alu repeats [31,32] and DNase I hypersensitive sites [33] and is directed by LEDGF/p75, a cellular binding partner of lentiviral integrases [34, 35]. Gamma-retroviral vectors have been shown to integrate preferentially around promoters and CpG islands [36]. The integration profiles of RV and LV have been studied in greater detail since it became clear that integration preferences can influence the potential vector genotoxicity for gene therapy applications. The main differences between stimulation protocols used to transduce $CD34^+$ haematopoietic/progenitor cells (HSCs) cells might influence the integration profile observed with patients. However, an *in vitro* mapping study of RV and LV integration in transduced human HSCs revealed that RV but not LV hot spots were highly enriched in proto-oncogenes, cancer-associated common insertion sites and growth-controlling genes, suggesting that LV have a lower propensity for integrating in potentially dangerous regions within the human genome [37]. Based on results obtained using a tumor-prone murine model, which served as an *in vivo* genotoxicity assay for a panel of chimeric vectors, differences in the oncogenic potential of γRV and LV could also be explained by the observed preferential targeting of cancer genes by γRV [38]. The study also revealed a determinant role for LTRs in the integrating vector's genotoxic potential, supporting the choice of self-inactivating LTRs in viral vector design (*see infra*).

Based on intrinsic integration site selection, HIV-1 derived LV appear to have an inherently safer profile than RV in relation to oncogenesis. However, LV integration into transcriptionally active genes still represents an enhanced

risk with the potential to cause a loss of heterozygosity and/or the loss of normal tumor suppressor gene functions. In addition, the risk associated to transactivation of neighbouring genome sequences must also be considered as *in vitro* studies suggest vectors' enhancer-promoter elements have a determinant role in the insertional transformation capacity of LV [18, 39-41]. Because there was a three year latency period before adverse events became clinically evident in the clinical trials using gamma-retroviral vector gene transfer [12], the relative safety of HIV-derived LV will not be demonstrated until a longer follow-up period has lapsed and more patients have been treated and studied.

An additional potential adverse event to consider in association with LV use is the mobilization and subsequent spread of mobilized vector particles to previously untransduced (non-target) cells or tissues. Mobilization could occur in vectors that retain their full LTR and when packaging proteins are provided in *trans*, similar to that observed during HIV infection or infection with other wild-type lentiviruses. LV can be rescued *in vitro* using wild-type HIV in T cell lines and primary human lymphocytes [42, 43]. More recent findings during the first phase I clinical trial, using LV for transferring anti-HIV-1 genes in chronic HIV infected patients, also detected transient mobilization of conditionally replicating HIV-derived LV. This was characterized by the presence of vector RNA in the plasma of four out of five patients. However, it should be noted that in this particular study the vector did not contain the enhancer-deleted SIN LTR commonly used in LV studies *(see infra)*. While vector spread is a significant concern for most gene therapy applications, vector mobilization in the setting of this clinical trial was considered beneficial to permit vector (anti-HIV-1 gene transfer) spread to additional T cells [44]. However, though the spectrum of cells infected by wild-type HIV is narrow, these findings do raise concerns relative to vector mobilization in patients treated with LV gene therapy who subsequently are infected with HIV.

3. SAFETY IMPROVEMENTS IN THE DESIGN OF HIV-1 DERIVED LENTIVIRAL VECTORS

In addition to the *gag*, *pol* and *env* structural genes common to all retroviruses, HIV-1 contains two regulatory genes, *tat* and *rev*, essential for virus replication and four accessory genes, *vif*, *vpr*, *vpu* and *nef* that while dispensable for virus growth *in vitro* are critical for *in vivo* replication and pathogenesis. As discussed above, the lentiviral genome is flanked by LTRs that play an important role in virus replication and gene transcription (Fig. **1**). The design of viral vectors derived from wild-type homologues first requires that the *cis*-acting sequences directing viral genome transfer (provided by a transfer vector) be separated from the *trans*-acting sequences encoding viral structural proteins (provided

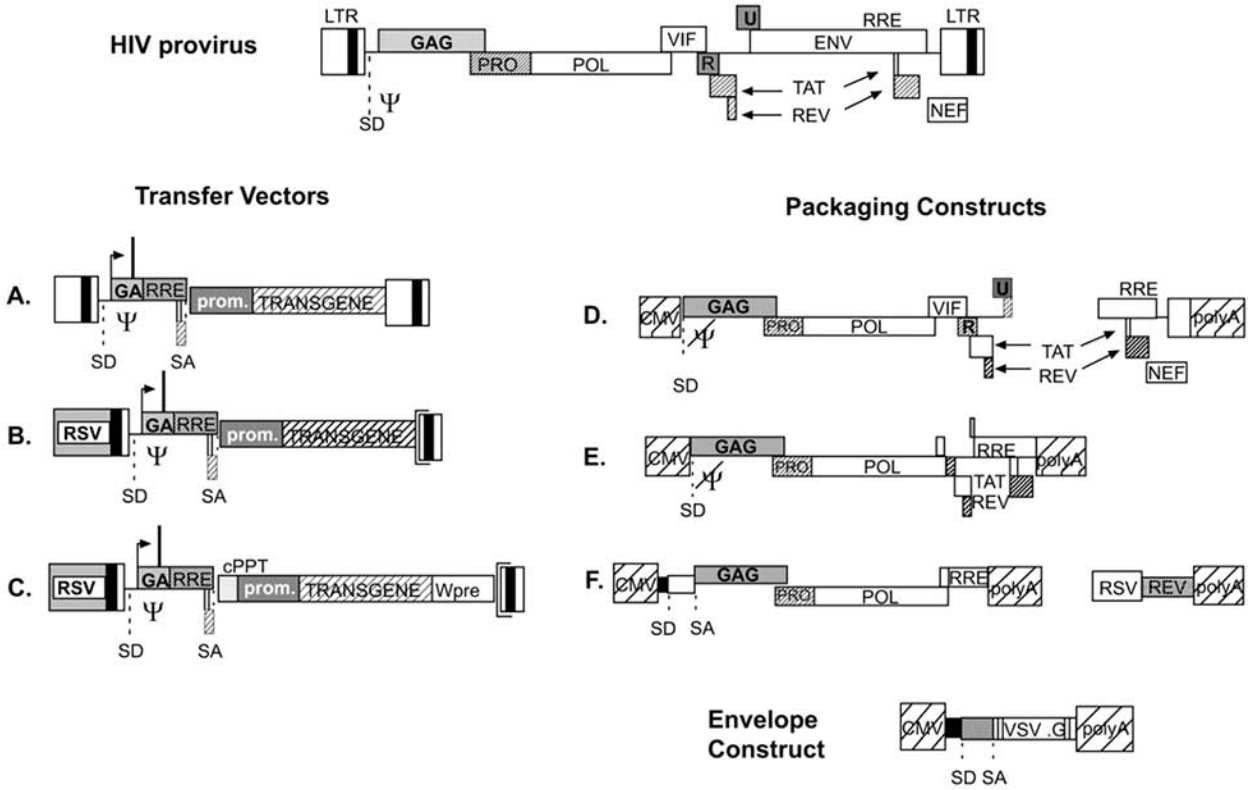

Fig. (1). Schematic representation of HIV provirus with on the left typical HIV-1 derived transfer vectors : wild-type **(A)**, Self-Inactivating (SIN) **(B)** and Improved SIN Transfer Vector **(C)**; on the right typical HIV-1 derived packaging constructs: first **(D)**, second **(E)** and third **(F)** generation packaging constructs. The envelope construct is unrelated to HIV-1 and is used to pseudotype the vector (E). **LTR**, long terminal repeats; **SD**, splice donor; **GA**, portion of the HIV-1 *gag* gene with a closed reading frame; **CMV**, cytomegalovirus strong promoter; **prom.,** internal promoter; **polyA**, polyadenylation signal; **RSV**, rous sarcoma virus promoter; **SA**, splice acceptor; **ψ**, packaging signal; **RRE**, Rev responsive element; **VSV-G**, vesicular stomatitis virus G protein; **WPRE**, post-transcriptional regulatory element of the woodchuck hepatitis virus; **cPPT**, central polypurine tract.

by the packaging construct). This approach produces LV transfer vectors containing a transgene expression cassette with an internal promoter that can drive transgene transcription flanked by the two viral LTRs. The packaging construct is characterized by the substitution of the viral LTRs at the 5' and 3' positions by a strong promoter and a polyadenylation signal, respectively. Several additional strategies have been employed to further improve the biosafety of LV:

3.1. Pseudotyping

Wild-type HIV-1 (and HIV-2) infection is restricted to human cells expressing the CD4 receptor (mainly helper T cells) in conjunction with the co-receptors CCR5 and CXCR4. One of the first developments in LV design was replacement of the natural HIV envelope gene with the vesicular stomatitis virus glycoprotein (VSV-G) gene, which greatly broadened cellular tropism (to most if not all mammalian cells) and because of the improved stability permitted its concentration by ultracentrifugation [45]. This 'first generation' of LV excludes formation of wild-type HIV even in the unlikely event that recombination between the packaging and the transfer plasmid occurred. Of note, it has also been reported that VSV-G pseudotyped LV particles can be inactivated by human serum when produced in human cells [46]. However, as high mutation rate of potential RCL could confer complement resistance, it is unlikely that RCL would have reduced capacity of replication in human serum. In addition to the pantropic nature of VSV-G pseudotyped LV, VSV-G pseudotyped LV preparations seem also heavily contaminated with tubulovesicular structures of cellular origin, which carry nucleic acids, including the DNA plasmids originally used for plasmid LV generation. These features may need further consideration when using recombinant LV in gene therapy and research [47].

Studies have shown that other viral glycoproteins are also suitable for pseudotyping HIV particles [48, 49]. For example, replication-defective LV pseudotyped with the ecotropic envelope protein from murine leukaemia viruses are appreciated because they can be concentrated by ultracentrifugation or ultrafiltration, albeit at lower speeds than VSV-G, and they have demonstrated increased biosafety in preclinical mouse models [50].

Other envelopes used for pseudotyping LV include the chimeric envelope glycoproteins RD114-TR and GALV-TR, made from the extracellular and transmembrane domains of feline leukaemia virus RD114 and gibbon ape leukaemia virus, respectively and the cytoplasmic tail of the murine leukaemia virus amphotropic envelope (TR) [51]. LV particles pseudotyped with these envelopes can also be concentrated by centrifugation. Moreover, unlike VSV-G pseudotyped LV, these vectors do not transduce murine embryonic fibroblasts, which are commonly used to support the undifferentiated state of different human embryonic stem cells [52]. Finally, analysis of the RD114-TR pseudotyped LV's transduction capabilities suggests they could be the envelope of choice for clinical studies designed to safely and efficiently genetically modify human haematopoietic stem cells [53]. Additional examples of lentiviral pseudotypes and a comparison in their titers, viral particle stability, toxicity and host-cell specificity has previously been reviewed by Reiser *et al.* [54].

3.2. Accessory Gene Removal

Removal of viral genes from the packaging construct that are not absolutely required for gene transfer but are important for HIV-1 virulence and pathogenicity such as the accessory genes (*vif*, *vpr*, *vpu* and *nef*), produced the "second generation" vectors, referred to as the 'multiply attenuated' vectors [55] (Table **1**). Elimination of the HIV *tat* gene was a further step in their improved biosafety. The Tat protein is a potent transcriptional activator responsible for the high replication rate of HIV. Tat not only affects the vector containing cells in which it is produced but it is also secreted and taken up by bystander uninfected cells. While intracellular Tat has been shown to alter the expression of both viral and cellular genes, including a number of important cytokines, it has also been shown that extracellular Tat is immunosuppressive and can induce apoptosis. Finally, there is evidence that exogenous Tat is involved in AIDS-associated pathologies such as Kaposi's sarcoma and HIV-associated dementia. Because Tat is important for efficient transcription of the vector genome during vector production, strong constitutive promoters were fused upstream from the HIV-LTR in the transfer vector to ensure production of sufficient RNA for efficient encapsidation and transfer by vector particles in the absence of *tat* [56]. However, it should be noted that this strategy of eliminating the *tat* gene is not always adopted in the design of more recent production systems.

3.3. Reducing the Probability of Homologous Recombination

RCL generation is believed to occur through homologous recombination between overlapping sequences. This risk has been minimized both by removing non-essential genes (described above) and by separating functional viral components into separate expression plasmids, which produced the so-called 'third generation' packaging system [56, 57]. The rationale for separating elements in different expression plasmids is based on the reduced probability that two, three or even four recombinations will occur. In addition to the *tat* gene deletion, Rev protein expression is directed by a separate non-overlapping expression construct. Regions of homology between the vector and packaging construct were further reduced by constructing Rev-independent codon-optimized gag-pol expression plasmids, which for HIV-1 are also devoid of the 5' untranslated region [58, 59]. Efforts to improve the strategy of separating genes between different plasmids lead researchers to design novel lentiviral packaging systems where not only Gag is supplied separately from Pol [60] but Protease (PR) expression was also independent [61]. Separating the overlapping gag-pol structure on two plasmids prevents the formation of functional gag-pol structures, which are essential for vector mobilization [62]. However, a drawback of this strategy is that both transducibility and production efficiencies are increasingly challenged by the number of plasmids required to produce the full complement of viral genes.

Table 1. Comparison of Lentiviral Production Systems (*)

	First generation [40]	Second generation [49]	SIN third generation [50, 60] (1)	Lenti-X™ [56] (2)	Translentiviral™ [56] (3)	Super-split system [55]
Number of plasmids	3	3	4	6	6	7
Deletion in the 3'LTR ("self-inactivation")	No	No	Yes	No	Yes	Yes
Number of packaging plasmids containing HIV genes	1	1	2	3	3	6
Accessory genes *vif*, *vpr*, *vpu*, *nef*	All present	All absent	All absent	All absent, except for non functional *vpr* that is fused to coding sequence of Pol and is packed into the particles formed as a fusion protein with RT and IN	All absent, except for non functional *vpr that* is fused to coding sequence of Pol and is packed into the particles formed as a fusion protein with RT and IN	*Vpu* and *nef* are absent, *vpr* is fused to PR and RT/IN-Vif functions which are delivered on separate plasmids
Sequences encoding Tat and Rev protein	Tat and Rev are present on single packaging construct	Tat and Rev are present on single packaging construct	Tat is absent, Rev is expressed from a separate, non-overlapping construct	Tat and Rev are present on a single separate construct	Tat and Rev are present on a single separate construct	Tat and Rev are present on two separate constructs
Overlapping Gag and Pol polyprotein structures	On the same plasmid	On the same plasmid	On the same plasmid	Split over 2 plasmids	Split over 2 plasmids	Split over 3 plasmids
Requirement for RCL formation	2 recombinations	3 recombinations	4 recombinations, between plasmids without homology and pick up of a promoter to complement 'Sin' deletion	4 recombinations, between plasmids without homology, recombination with transfer-vector, repair of point mutations, pick up of a promoter function to allow expression of Tat, Rev, Gag and Pro	4 recombinations, between plasmids without homology, recombination with transfer-vector, repair of point mutations, pick up of a promoter function to allow expression of Tat, Rev, Gag and Pro	more than 4 recombinations

(*) lentiviral expression systems described in this table all have a separate construct expressing Vesicular Stomatitis Virus G glycoprotein (VSV-G) instead of the *env* gene encoding the HIV-1 envelope.

[40] Burns JC *et al*., 1993, [49] Zufferey R. *et al.,* 1997, [50] Dull *et al.,* 1998, [55] Westerman KA *et al.,* 2007, [56] Kappes *et al.,* 2001,[60] Zufferey *et al.,* 1998.

(1) Commercially available : ViraPower ™ from Invitrogen™

(2) Commercially available : Lenti-X™ Expression System from Clontech Laboratories (3) Commercially available : Trans-Lentiviral ™ from Open Biosystems

Another effort designed to reduce the probability of homologous recombination involves the creation of hybrid vectors obtained through the cross-packaging of transfer vectors from one virus with the packaging machinery from a second virus. The rationale behind this approach was that the difference in sequence between the viruses could be sufficient to curtail recombination whereas their similarity would still ensure the formation of a functional particle. Based on this approach, the packaging of HIV-1 genomes using SIV particles [63] or HIV-1/HIV-2 chimaeras [64, 65] has been investigated.

3.4. Self-Inactivating Transfer Vectors

Another strategy that can improve the safety of LV involves deletion of the promoter and enhancer elements located in the transfer vector's 3'LTR U3 region. Vectors with

this deletion are called self-inactivating (SIN) vectors because the U3 deletion is copied from the 3'LTR to the 5' LTR during reverse transcription thereby producing integrating vectors that contain U3 modified LTRs with reduced promoter activity (10% of original activity). The usefulness of this modification is illustrated by the fact that it addresses most LV biosafety issues, including the risk of: (i) recombination between the transfer vector and packaging constructs (ii) insertional oncogenesis when LTRs are used, and (iii) mobilization of HIV-1-based transfer vectors from transduced target cells by subsequent (or prior) infection with wild-type HIV-1 [66, 67]. The latter two issues require more consideration. First, although deletion of the U3 region elements can reduce the risk associated with enhanced expression of genes surrounding the integrated vector genome, the internal enhancer and promoter required for transgene expression continue to pose a relevant risk of transactivation and therefore need to be carefully chosen. Cellular (internal) promoters are weaker insertional mutagens compared to retroviral promoters [68-70] and using lineage or tissue specific promoters can potentially increase biosafety. Second, although the risk of mobilization is reduced, studies have shown that mobilization is not totally eliminated in the SIN vectors [71-73].

3.5. Improved Efficacy of LV

Additional elements are currently routinely being introduced in LV constructs, not to improve vector safety but to increase transduction efficiency. These elements include but are not limited to: (i) the lentiviral central polypurine tract (cPPT) thought to facilitate reverse transcription and nuclear import of the lentiviral pre-integration complex prior to vector integration [72, 74, 75] and (ii) the Woodchuck hepatitis posttranscriptional regulatory element (WPRE) that has been shown to increase LV titers and expression of RVs and LV by presumably improving mRNA half-life, export and polyadenylation [76-78]. Previously, remaining safety concerns addressed the presence of the X protein ORF within the PRE. However, PRE variants devoid of the X protein and their promoter are now constructed which still preserve their beneficial effect on titer and transgene expression [77].

Upstream polyadenylation enhancer elements have also been introduced to increase recognition of the correct polyA site [79]. The addition of these elements may also increase biosafety because normally up to 10% of the transcripts are not correctly terminated at the 3'LTR, which could lead to a read-through into neighbouring (onco) genes [80, 81].

In conclusion, the risk of RCL production has been considerably reduced by the development of safer virus production systems with no reported incidents of RCL generation. The low probability of RCL formation with LV appears to be substantiated by production protocols designed to produce clinical grade LV batches free from RCL. Within this context, PCR and sensitive culture based methods for RCL detection have been developed and confirmed the absence of RCL in large production batches [82, 83].

4. COMPARISON OF LENTIVIRAL VECTOR PRODUCTION SYSTEMS

Most of the LV production systems employ at least three plasmids: an envelope construct, one or several helper constructs encoding Gag and Pol viral proteins and a transfer vector. First and second generation vectors are considered less safe because they contain more original HIV sequences compared with third generation LV, which theoretically decrease the number of recombination events necessary to form RCL. However, following the development of the so called 'third generation' vectors, the design of more recent LV production systems has not always been associated with the removal of additional HIV sequences nor the use of a self-inactivating transfer vector. In addition, choosing the most appropriate LV production system in terms of biosafety cannot simply be based on the quantitative comparison of RCL generation probability because to date no RCL formation has been reported with any LV. Hence, the most appropriate LV production system for a given application should be chosen, from the biosafety point of view, on qualitative attributes. Table **1** illustrates the properties of some LV production systems.

The Lenti-X ™, the Translentiviral™ as well as the Super-split production systems all include more HIV genes than 'third generation' systems. Although preference should be given to the use of LV production systems that contain a minimum of HIV sequences, the probability of RCL generation using the non-SIN Lenti-X ™ production system is assumed to be the same or lower than the third generation SIN vectors. The rationale is that the Lenti-X ™, the Translentiviral ™ as well as the Super-split production systems all have their Gag-Pol structure split between at least two plasmids, which prevents functional Gag-Pol structures that are essential for vector mobilization from forming.

The design features of the Lenti-X ™ production system inherently have two theoretical disadvantages. First, in comparison with a SIN vector, the Lenti-X production system actually has an increased probability of vector mobilization: if a wild-type virus infects the transduced host cell, subsequent Tat protein expression can activate the lentiviral system. However, this risk can be considerably reduced by pretesting the host cells for relevant wild-type viruses and implementing measures that prevent their inadvertent contamination.

Second, when using a non-SIN LV production system, the risk of transcriptional effects due to insertional mutagenesis must be considered. Although the HIV LTR is Tat-dependent (Tat is not co-packaged in the vector particles) the LTR is not completely inactive in the absence of Tat. Therefore, the risk of gene activation using a non-SIN transfer vector could be considered higher compared to a SIN transfer vector. As discussed above, recent studies have shown that LTRs are major determinants of genotoxicity and this further supports the use of SIN viral vectors [38, 84]. Another caveat separate from the LTR that also must be considered are the potential effects from the internal promoter driving transgene expression. Most internal promoters carry potent enhancer elements that potentially could counteract the effectiveness of transcript termination, normally achieved by the naturally weak lentiviral polyA and improved by insertion of the Woodchuck hepatitis posttranscriptional regulatory element (WPRE) [76-78] or upstream polyadenylation enhancer elements (USEs) [79].

Other novel LV designs include a point-mutated integrase with the so-called class I mutation, where virus integration is abolished while the other roles of integrase remain unaffected. Such integration-defective lentiviral vectors (IDLV) have increased levels of LV DNA circles. Actually, these LV DNA circles are also generated during normal lentiviral infection, yet they have traditionally been considered as dead-end products of reverse transcription. Taking advantage of these episomal circles, IDLV have recently emerged as novel and efficient gene carriers, facilitating high levels of transient expression from linear and circular DNA forms [85-88]. Recently, the episomal nature of the integration-defective vectors was also exploited as a template source for high-efficiency gene correction through homologous recombination in human cell lines and embryonic stem cells [89, 90].

Searching for a way to alter the lentiviral integration profile, studies found that IDLV could be integrated in a site-directed manner by replacing the normal viral integration machinery with a non-viral mediator of integration, such as Flp, a yeast recombinase [91]. This approach decreases the chance of insertional mutagenesis and could pave the way for future applications of integrase deficient LV that target gene insertion to predetermined insertion sites. However, one drawback is that a small percentage of uncontrolled LV DNA circle integrations occur, possibly through illegitimate integration mechanisms [92]. Further studies are needed to demonstrate that the long term gene expression observed with IDLV is not due to their illegitimate integration. An alternative strategy for overriding the natural integration profile with a bias to transcriptionally active genes, recently proposed by the same group, involves constructing a hybrid vector system combining IDLV with a non-viral Sleeping Beauty (SB) transposon vector encoding SB transposase [93].

5. RISK ASSESSMENT OF ACTIVITIES INVOLVING LV USE

Aside from the identification of hazards intrinsic to the LV itself, a thorough risk assessment must also consider the conditions whereby these vectors will be handled. Typical laboratory procedures include: (i) manipulating and handling LV transduced cell cultures, (ii) handling LV suspensions, (iii) *in vivo* experimental research involving laboratory animals that are inoculated with LV suspensions or LV transduced cells, and (iv) vector preparation. Exposure risks must be identified for each procedure with particular attention paid to potential inadvertent host contamination during *in vivo* experiments or the presence of endogenous retroviral sequences potentially present in non-established cell cultures.

5.1. Handling LV Transduced Cell Cultures

Animal cells have limited survival if they are not maintained under proper culture conditions. Therefore, the major biological hazard associated with *in vitro* manipulation of transduced cells is the potential for accidental inoculation of a lab worker. Integration of LV in the cellular genome, which is associated with a risk of insertional mutagenesis or transactivation of neighbouring genes as well as the possible stable expression of hazardous transgenes, can potentially confer an expanded life-span, an increased risk of tumour formation or other harmful effects to the transduced cells. However, the real extent of these putative harmful effects from LV transduced cell inoculation in a host organism is difficult to predict. The histocompatibility mismatch between the transduced cells and the host organism remains a major obstacle in their survival and expansion because they are under the constant pressure of recognition and destruction by the immune response in a non-immunocompromized host. This is also one of the principal reasons why culturing cells that originate from the individuals working in the laboratory should be strictly prohibited.

5.2. Handling LV Suspensions

The handling of LV suspensions, in particular high titer stocks, increases the possible accidental exposure of the lab worker and therefore is considered to be an activity associated with higher risks. The major potential hazard is infection of the researcher by parenteral inoculation (e.g. needle stick accidents) because after accidental exposure, LV can potentially integrate in the infected host cell genome. This could not only produce hazards linked with insertional (in)activation or transactivation of genes but might also result in permanent transgene expression with the associated harmful effects based on the gene product expressed. While the risks pertaining to hazardous transgenes will not be covered in this review, they remain an important element requiring careful consideration. Specific classes of genes, including oncogenes, cytokine coding genes and virulence genes, are inherently associated with a higher risk. Short hairpin RNA cassettes that are routinely introduced by LV to mediate gene silencing can also knockdown tumor suppressor genes. Given the wide variety of potential transgenes or shRNA cassettes, risk assessment of LV carrying transgenes remains a case-by-case approach that needs to be carefully performed.

5.3. *In Vivo* Studies

After accidental or deliberate LV infection of the host, a theoretical risk of recombination remains for vector sequences as well as sequences derived from wild-type viruses present in the host, which can potentially have adverse effects. For example, recent observations in an *in vivo* ovine model found a direct deleterious *in vivo* effect associated with recombination between a gibbon ape leukaemia virus (GaLV)- pseudotyped MLV-derived retroviral vector and bovine leukaemia virus, a leukaemogenic complex retrovirus that infects cattle and sheep [94].

The use of HIV-derived LV in murine models circumvents the potential risk of adventitious wild-type viruses as rodents are not permissive for HIV infection. Furthermore some studies indicate that the (adventitious) presence of gamma-retroviruses in the host system hardly has an adverse effect. Cross-packaging between gamma-retroviruses and lentiviruses has not been observed in cultured cells [95] and even in transgenic mice with each cell carrying the HIV proviral genome, Southern blot analysis of tissues failed to detect the presence of free HIV proviral containing sequences [96]. The potential for generating replication-competent len-

tiviruses in an *in vivo* xenotransplantation system has also been investigated. Assessment of adverse event risks in 149 immune-deficient mice transplanted with lentiviral transduced human haematopoietic stem and progenitor cells [97] found no significant vector associated adverse events and no detectable HIV p24 antigen in their sera during several months of follow up. However, despite these 'reassuring' findings, the authors stressed that *in vivo* assessment over a longer period of time, compared to this limited murine study, is likely necessary.

Particular attention should be paid when the host is permissive for lentiviral infection because there is an increased risk for the mobilization of lentiviral vectors or complementation that in turn increases the potential for RCL formation. As mentioned earlier, findings of the first clinical trial using LV have actually revealed mobilization of lentiviral vector sequences in patients with chronic HIV infection [39]. Another issue is the mobilization of LV in patients who, after gene therapy, acquire an HIV infection.

5.4. Endogenous Retroviral Sequences

The use of packaging cell lines for vector preparation and transduction of specific target cells with LV potentially initiates interactions with endogenous retroviral (ERV) sequences present in the host cells or packaging cell lines. ERV are remnants of ancient germ line infections by exogenous retroviruses that have integrated into the genome. Some may have moved and spread around the host genome by retrotransposition. It is now recognized that some ERV are able to interact with exogenous counterparts, including retroviral vectors, through a variety of mechanisms including recombination and transactivation [10, 98, 99]. Proteins provided *in trans* by retroviral vectors can also induce mobilization of otherwise inactive endogenous retroviruses [100] or co-packaging of ERVs can result in the unwanted transfer of their sequences to target cells and the potential recombination with retroviral vectors or the cellular genome [101]. Likewise, the use of LV in clinical trials warrants a consideration of the potential risks associated with the presence of human endogenous retroviruses (HERVs). HERVs make up as much as 8% of the human genome [102] making recombination between these endogenous sequences and viral vectors theoretically likely.

In vitro studies investigating the incorporation of HERVs in murine leukaemia virus (MLV) vector particles at least suggested there is inefficient recognition and packaging of the HERVs by the MLV assembly machinery [103]. Data from a recent study suggested that an HIV-1 derived LV system co-packaged the HERV sequences to a lesser degree than MLV-based systems [104]. The authors partly explained this observation by the fact that no lentivirus related HERVs are present in the human genome in contrast to the considerable amount of MLV related HERV sequences. Still, some HERVs have sequences that are recognized by HIV-1 Rev after HIV-1 infection in a permissive cell, thereby promoting nuclear export of the HERV transcripts in these cells [105]. Moreover, RNA derived from HERVs is detectable in the plasma of HIV-1-infected individuals [106-108] supporting the view that significant protein coding capacity and activity potential still exist for these endogenous retroviruses [109, 110]. The proportion of HIV packaged HERVs compared with released HIV RNA remains unclear and it is accepted that different forms of cellular stress can induce non-specific expression of HERVs. However, the recent observations indicate there might be a need to address the possibility that HERV are a source of functional lentiviral sequences capable of recombining with HIV-derived LV.

It is too soon to establish an LV safety profile for human gene therapy. Several clinical trials using LV are still ongoing and need further follow-up to detect any adverse effects [111]. Since the first clinical applications using LV targeted human T lymphocytes for the treatment of HIV infection (e.g. expression of anti-HIV RNAs) or cancer, the field of LV-mediated gene therapy has recently been expanded with trials targeting autologous CD34+ haematopoietic stem/ progenitor cells for the treatment of demyelinating disorders including X-linked adrenoleukodistrophy haemoglobinopathies or stem cell defects such as sickle cell anaemia, thalassemia major or Wiskott-Aldrich syndrome (currently under consideration). The outcome from these clinical trials will most certainly provide valuable information concerning the clinical suitability and safety profile of LV.

6. COMPARISON OF BIOSAFETY RECOMMENDATIONS

Many of the international guidelines and recommendations for safe handling and manipulating hazardous biological agents do not address LV specifically. More attention is given to risk assessment for retroviral vectors in general, emphasizing that the control measures implemented should be determined on a case-by-case basis. Some guidelines provide a few recommendations on the use of LV. For instance, the UK Health and Safety Executive specifies that activities involving defective lentiviruses can be classified as class 1 as long as they have restricted tropism (i.e. are unlikely to infect human cells) and a low probability of RCL generation (as for third generation ecotropic retroviruses) [112]. In Germany, LV with ecotropic envelopes (i.e. infect only murine cells) can be manipulated using BSL-1 conditions while the production and transduction of VSV-G pseudotyped LV must be handled using BSL-2 procedures, unless the cells have been passed or washed twice and RCL negativity has been demonstrated [113]. According to Belgian regulations, the production and use of HIV-1 derived LV should at least be conducted in a biosafety level 2 (BSL-2) laboratory. A BSL-3 is required if large quantities are used or transgene sequences encode potentially hazardous gene products. These recommendations do not take into account recent properties of LV design, including the U3 deletion of the 3'LTR (SIN transfer vector), the number of plasmids during production or the presence of HIV accessory genes. Therefore, these new features warrant a closer look, in particular for HIV-1-derived vectors where considerable progress in LV design and safety has occurred.

Table **2** provides an overview of the most recent recommendations and guidelines that specifically address LV in contained use activities. This summary illustrates that a comparison between established guidelines is not straightforward and is hampered by several factors. First, the inherent LV design features considered important for containment

Table 2. Biosafety Recommendations and Guidelines Addressing Lentiviral Vector Manipulations

Type of Manipulation	Level of Containment	Additional Measures or Conditions	Reference No.
In vitro			
Manipulation of cells transduced with SIN vectors of third generation	BSL 1	Conditions on the inoculum (see Table **4**)	[115,116]
Research using systems with vector packaging functions on more than two plasmids	BSL 2		[119]
Manipulation/production of SIN-vectors devoid of regulatory proteins Vpr, Vpu, Vif and Nef, volume < 200 ml + manipulation of cells transduced with such vector	BSL 2	Gloves, PPE [a]	[114]
Manipulation of cells transduced with non-SIN-vectors or vectors not devoid of regulatory proteins Vpr, Vpu, Vif and Nef	BSL 2	p24 Elisa test is negative	[114]
Research using Lentivirus vector with vector packaging functions on 2 plasmids	BSL 2 enhanced	Attention to sharp tools (use of safety needles), PPE [a] when producing large volumes (> 10L)	[119]
Manipulation/production of non-SIN-vectors or vectors not devoid of regulatory proteins Vpr, Vpu, Vif and Nef or use of vector in volumes > 200 ml	BSL 3		[114]
In vivo			
Housing of animals inoculated with LV with vector packaging functions on more than two plasmids, 1-7 days after inoculation	BSL 1	Animal is not permissive for lentiviral infection, site of inoculation has been cleaned, bedding is changed	[119]
Housing of animals inoculated with i) SIN vector devoid of regulatory proteins Vpr, Vpu, Vif and Nef or ii) cells transduced with this vector	BSL 1	Used vectors show negative P24 Elisa test when transduced in C8166 cells	[114]
Inoculation of animals with i) non- SIN vector or vector not devoid of regulatory proteins Vpr,Vpu, Vif and Nef or ii) cells transduced with this vector	BSL 1	p24 Elisa test is negative	[113]
Inoculation of animals with systems with vector packaging functions on more than two plasmids	BSL 2		[119,see also 118]
Inoculation of animals with i) SIN vector devoid of regulatory proteins Vpr, Vpu, Vif and Nef or ii) cells transduced with this vector	BSL 2	With use of biosafety cabinet type II	[114,see also 118]
Transplantation of transduced cells in primates using SIN 'third generation' lentiviral vectors	BSL 2	Conditions on inoculum dose to minimize presence of free vector particles.	[117]
Inoculation of animals with vector with packaging functions on 2 plasmids	BSL 3	Minimize the risk of autoinoculation	[119]
Animals engrafted with human cells or animals permissive for lentivral replication	BSL 3	Attention to sharp tools (use of blunt-end needles), PPE [a]	[119]

a : intended to reduce potential for mucosal exposure.

PPE: Personal Protective Equipment

Overview of biosafety recommendations and guidelines specifically addressing the risk assessment of lentiviral vectors. Minimal containment requirements are given for LV for which the nature of the transgene insert poses no additional risks and the production of the vectors occurs on a laboratory scale.

level determination differ among several guidelines. For example, the French "Commission de Génie Génétique" [114] poses less stringent measures for LV containing a SIN transfer vector devoid of the regulatory proteins Vpr, Vpu, Vif and Nef, thus necessitating LV production systems that use a minimum of three plasmid constructs. In the Netherlands, COGEM [115-118] recommends that a down-scaling of containment measures is acceptable for SIN "third generation" LV, which are devoid of regulatory proteins and produced using a minimum of four plasmid constructs. Another factor hindering a comparison between recommendations is their lack of harmony in the minimal requirements for containment facilities. The ACGM (UK) guidelines for example, indicate that if specific measures are necessary to control lab worker exposure to the vector (e.g. gloves, a microbiological

biosafety cabinet, restricted access) then the activity should be assigned to risk class 2 [112].

In general, guidelines recommend a BSL-2 with particular attention for sharp tools. We think indeed that the implementation of BSL-2 measures are adequate for the production or handling of most replication-defective LV, unless large volumes are exceeded (according to NIH this is >10L production volumes, [119]) or additional risks are posed by the transgene itself. Table **3** describes work practices and safety equipment that are in standard use for manipulating LV in a BSL-2 facility. These recommendations focus on the importance of preventing biological risks and taking adequate measures following accidental exposure. In this respect, the current guidelines for managing occupational exposure to HIV rely on the use of antivirals. Ideally, it is recommended that post-exposure prophylaxis (PEP) be administered within 1 h following a needle-stick injury to minimize the risk of developing HIV seropositivity. Antivirals such as AZT effectively inhibit the viral reverse transcriptase from forming a DNA copy from the viral RNA, leading to the proposal of implementing a PEP protocol for HIV-derived LV to minimize the biological hazards associated with inadvertent transduction when using potentially harmful transgenes [120]. PEP treatment needs to be prescribed by an occupational health physician because antiretroviral drugs are frequently not well tolerated and who therefore must determine whether treatment is necessary based on the seriousness of the exposure and the biological hazards associated with the expressed transgene.

The absolute requirement for working in a BSL-2 may be questionable when the activities are limited to handling of cell lines transduced with replication- defective LV. It is generally accepted that defective LV, whose probability of generating RCL is considered negligible, barely confer any additional risks to transduced cells. The requirement for a BSL-2 facility may actually be based on the overall risks associated with the intrinsic properties of the mammalian cells being used. In Belgium for example, regional legislation for the contained use of pathogens and/or genetically modified organisms recommends that BSL-2 containment be used for the manipulation of primary cells of primate or human origin, irrespective of their transduction with LV. Alternatively, well characterized and certified cell lines carrying no increased risk resulting from contaminating pathogens can be manipulated in BSL-1 containment using a biosafety cabinet (BSC) type II if the cell lines are of human or primate origin [121]. Table **4** lists a number of conditions whereby we think that LV transduced cells can be handled using BSL-1 containment. This list is based, in part, on the recommendations by COGEM for manipulating transduced mammalian cells in a BSL-1 facility. These guidelines focus more specifically on efforts to reduce the probability of free lentiviral particles being present after cell transduction. For HIV-derived LV, this reduction can be achieved by washing the transduced cells with growth medium, inactivating free LV by treating the transduced cells with trypsin or human serum [122, 123] or increasing the incubation time since the half life of lentiviruses is 10h at 37°C [124]. It is also been shown that dendritic cells and monocyte-derived macrophages can internalize HIV-1 which can maintain its infectious form for several months [125-127]. Thus, simply increasing the number of washings or prolonging the incubation time will not eliminate free virus particles that can potentially still be released after a prolonged period of time. Transduced dentritic cells and macrophages therefore should be handled in a BSL-2.

Table 3. Specific Work Practices and Safety Equipment for the Manipulation of LV in a BSL-2 Facility

- The laboratory has at least one class II biological safety cabinet if open manipulations are performed. It is installed in order to avoid disturbing airflows equilibrium inside the work area. It is located away from doors, windows, room supply, exhaust air louvers, and from heavily travelled laboratory areas. It is controlled and certified when placed, after each moving and at least once a year.
- Disposable gloves are available for the personnel and must be worn when there is direct contact or possibility of contact with non-intact skin.
- Mask, eye protection or face shield are worn during procedures likely to create splashes or (to generate) aerosols.
- The use of needles and other sharp instruments should be avoided. If this is impossible, the instruments should be adequately managed to prevent or reduce the risk of percutaneous injuries.
- All manipulations likely to produce infectious aerosols or involving potential risks are conducted within a class II biological safety cabinet.
- Simultaneous manipulation of replication competent viruses or other vector systems in the same class II biosafety cabinet is prohibited.
- Use of a horizontal airflow cabinet is prohibited for the manipulation of pathogens and/or genetically modified (micro)-organisms.
- Work surfaces are cleaned and decontaminated with an appropriate disinfectant after work is finished and after any spill of biological material. Appropriate disinfectants for inactivating LV on surfaces include 1% sodium hypochlorite, 2% alkaline glutaraldehyde or 70% ethanol .
- Directions for use of disinfectants are available for the personnel. Depending on the purpose, instructions precise the kind of disinfectant to use, its concentration, and contact time.
- Behaviour in case of accident is clearly posted in the laboratory. A post-exposure prophylaxis treatment protocol is set up and is started as soon as possible after prescription by the occupational health physician.
- Transport of LV or LV transduced cells within the facility must occur in a double packaging. Primary leak-proof receptacle must be packed in secondary packaging in such a way that, under normal transport, it must be unbreakable.
- Transport of LV or LV transduced cells outside the facility must occur in triple packaging according to the current requirements of the UN regulations regarding the transport of Dangerous Goods.

The risk assessment for LV transduced cells used under the conditions listed in Table **4** justifies compliance with the lowest containment level (BSL-1). However, it should be emphasized that implementation of good laboratory and microbiological practices (including restricted access) and use of a BSC is the usual standard operating procedure for cell culture in most laboratories. The added advantage of this is that if necessary, upgrading these laboratories to BSL-2 facilities normally should only require the implementation of simple additional safety measures.

Table 4. Conditions for the Manipulation of LV Transduced Cells in a BSL-1 Facility

- No primary cell cultures of primate or human origin.
- No cell lines requiring BSL-2.
- No dendritic cells as they have the capacity to internalize HIV-1.
- No increased risks from contaminating pathogens. In particular, the host cells should be free of HIV-1, HIV-2, *Human T-cell lymphotropic virus* type 1 (HTLV-1), HTLV-2, SIV or other relevant lentiviruses with tropism for the host cell.
- Cells have been transduced with replication defective LV particles produced by one of these systems:
  - VSV-G pseudotyped HIV-derived systems with vector packaging functions on minimally three plasmids (see third generation production systems) in combination with transfer vector devoid of the U3 3'LTR (SIN vector).
  - VSV-G pseudotyped HIV-derived systems with vector packaging functions on more than three plasmids.
  - Pseudotyped HIV-derived systems, not capable of infecting human cells, with vector packaging function on minimally three plasmids.
- The replication defective LV particles harbour no transgene or transgene cassette that could confer additional harm to the transduced cells for human health and the environment.
- Transduction of cells has been performed in a BSC, minimally 30 minutes after previous manipulations with replication competent viruses or other vector systems that have been conducted in the same BSC.
- Simultaneous manipulations with replication competent viruses or other vector systems have been avoided during the transduction of the cells in the BSC. The BSC has been properly cleaned and decontaminated with an appropriate disinfectant after each manipulation involving the use of viral vectors.
- Specific measures are adopted to reduce the presence of free lentiviral particles after transduction, i.e. (i) washing steps with growth medium (ii) inactivating free LV particles by treatment of transduced cells with trypsin or human serum (iii) increased time of cell culturing at 37°C.
- Working surfaces and/or instruments that have been in contact with the transduced cells are decontaminated with an appropriate disinfectant such as 1% sodium hypochlorite, 2% alkaline glutaraldehyde or 70% ethanol.

Many research activities include *in vivo* experiments where animals are injected with LV or LV transduced cells. Animals that are injected with LV transduced cells that meet the conditions listed in Table **4** can be housed at containment level BSL-1, however direct delivery of LV requires more care. The recombinant DNA advisory Committee (RAC) of the National Institutes of Health (NIH), COGEM, and ACGM all recommend BSL-2 containment for animals that are not permissive for lentiviral infection and the use of a BSC for initial delivery of the LV (Table **2**). Given the hazards posed by animal inoculation, particular attention must be paid to accidents with sharp tools. Viral injections that cannot be performed in a biosafety cabinet (e.g. stereotactic injection) should be conducted using BSL-2 conditions including gloves, goggles and surgical masks to reduce the possibility of mucosal exposure. The inoculation site should be thoroughly cleaned [119]. Animals that do not support replication of infectious HIV-1 that are injected with LV considered to have a negligible probability of RCL generation (e.g. use of SIN third generation vectors), can be housed in a BSL-1 after a period of time that ranges between 1 to 7 days.

Previously the declassification guidelines for animals treated with LV were based solely on the *in vitro* half-life (ranging from 10 to 24 hrs for a VSV-G pseudotyped LV in culture medium at 37°C). Accordingly, an animal injected with 10^6 active particles would stay at BSL-2 for at least 21 days, assuming that the vector half-life in the worst case scenario would be similar to the *in vitro* conditions. Recently shedding experiments have been performed using rats for intracranial and intravenous injection of high dose LV preparations (transducing units (TU) > 10^6 and 10^7 per dose respectively). p24 ELISA and titration of functional TUs using cell lines were assessed over time points post vector administration and found that p24 in the serum decreased to 50% of the injected dose within 20 minutes and was undetectable after two hours; during the same time period, no functional titers could be detected in the serum or urine (Toelen and Debyser, unpublished data). Similar data were obtained in a second lab [128]. Based on the observation of both research groups and taking into account the non-permessivity of rodents for lentivirus replication, we propose a set of containment criteria that should be implemened during and following the inoculation of rodents with third-generation SIN vectors (Table **5**).

Some recommendations rely on a negative p24 ELISA before accepting a lower containment level. However, the relevance of performing an RCL is questionable. Several efforts to demonstrate RCL generation in HIV-based LV preparations indicated that one can reasonably assume their frequency is very low when the latest generation of vectors are used [83,119, 120]. Thus, except when manufacturing LV particles for clinical use or during the declassification of containment measures, RCL testing will rarely offer any added value because their frequency is substantially lower than the detection threshold of the most sensitive tests currently available (1 RCL in 10^8 TU). RCL testing could actually increase the risk (compared to the test material) since each assay requires an appropriate positive control [119, 129]. RCL testing should therefore always be performed in a BSL-2 laboratory with adequate technological know-how.

Generally, the need for implementing additional containment measures over those required in a BSL-2 facility need to be based on the increased risk of LV exposure (including increased risk of RCL generation) and the identification of transgene-associated hazards.

Increased exposure may result from handling a large number of vector particles - high volumes and/or high titers - or from housing laboratory animals that are permissive to lentiviral infection. Most guidelines request higher levels of containment for animals grafted with human cells permissive for HIV-1 replication such as SCID-mice and *Mamu7* homozygous animals that lack TRIM5-mediated restriction of HIV-1 [130]. Handling first generation LV may also require the implementation of additional measures because these vectors are considered to be less safe and the probability of RCL is not negligible.

Table 5. Inoculation of Rodents (*) with Third Generation SIN Vectors and Subsequent Housing Requirements

- Inoculation is performed in a biosafety cabinet under BSL2 conditions. Appropriate personal protective equipment should be worn, including double gloves and protective gown. Animals are preferably anesthetized or puncture resistant gloves are worn.
- Safer, engineered needles or needless systems are used.
- The lesion/ injection site is decontaminated with 70 % ethanol before placing back the animal in the cage.
- All work surfaces are decontaminated with appropriate and validated disinfectants such as 1% sodium hypochlorite, 2% glutaraldehyde or 70% ethanol.
- Injected animals are kept within a BSL-2 facility (in individually ventilated cages (IVC) or filter-top cages) or within a BSL-1 facility (only in IVC cages), for minimally 72 hours post inoculation. The cages can only be opened under the biosafety cabinet. Animals are transferred to fresh cages, within a biosafety cabinet, not sooner than 3 days post-inoculation.
- During the first 72h post inoculation, waste materials such as bedding, faeces and urine require inactivation prior to disposal.
- Animals are not allowed to leave their cages (nor the BSL-2 facility in case of filter-top cages) during the first 24h post inoculation. After one day, animals are allowed to leave their cages for experiments (such as for imaging technologies) provided that p24 monitoring of shedding is negative. Contact surfaces are cleaned with 70% ethanol.
- 72h post inoculation, housing of rodents is allowed under BSL 1 conditions, p24 monitoring of shedding is not mandatory. Direct contact between the inoculated animal and other laboratory animals should be prohibited. Waste materials such as bedding, faeces and urine will not require inactivation prior to disposal.
- Staff should be given appropriate training and instruction on the procedures to be carried out. A set of standard operating procedures should be drawn-up, which should be read by all staff using the facility.

(*) excluding mice engrafted with human cells or mice lines permissive for HIV-1 replication (e.g. SCID mouse with human immune system)

LV are widely used as gene transfer vehicles, in part because they are characterized by stable expression of the transgene. A comprehensive risk assessment of LV should therefore also take into account the risk associated with the transgene products delivered by these vectors. A gene product may be intrinsically harmful (e.g. toxic properties) or could induce hazardous properties via its expression in transduced cells, dependent upon the genome integration site, promoter activity and expression of regulatory sequences governing expression. The risk assessment for transgenes, delivered or not by LV, is not straightforward. Because this issue merits more consideration than could be covered within the framework of this paper, we propose that interested readers consult literature specifically addressing this topic [131].

7. NON-HUMAN LENTIVIRUSES

Non-human lentiviruses are incapable of infecting human cells. However, this restriction may be overcome by pseudotyping the viral particles and/or altering viral promoter sequences, which has led to the development of LV from non-human lentiviruses. Several candidates, including simian immunodeficiency viruses (SIV) from various monkey species, feline immunodeficiency virus (FIV), equine infectious anaemia virus (EIAV), caprine arthritis/encephalitis virus (CAEV) and bovine Jembrana disease virus, have been proposed. The suitability and effectiveness of non-human LV in human cells depend on the capacity of the LTR to enable stable expression of the transgene in human cells and to allow production of viral particles in human producer cells. The consequences of human infection with LV derived from non-human primate- and non-primate-LV are unknown and thus safety concerns remain, particularly in association with risks from horizontal and cross-species transmission of any mobilized, recombined chimeric lentivirus. Noteworthy, a gene-based therapy for Parkinson's using EIAV is currently being evaluated in a Phase I/II trial [111, 132].

Generally BSL-1 is appropriate for Risk group 1 organisms. However, replication- defective vectors where a heterologous envelope (VSV-G) is used for packaging may require BSL-2 containment due to their increased ability of transducing human cells and risk for insertional mutagenesis.

CONCLUSIONS

The major risk associated with LV manipulation in the research laboratory resides in the inadvertent transduction of the lab worker. Upon transduction, the operator may be exposed to potential harmful effects coming from the transgene, insertional mutagenesis or the activation of neighbouring genes resulting from vector integration or the generation of replication competent viruses. In this respect, the effect of potentially deleterious transgenes merits particular attention since LV enable stable expression of these transgenes in dividing and non-dividing cells.

LV derived from HIV-1 may provoke biosafety concerns because of the well-known pathogenicity of the parental virus. However, considerable effort has been made to improve the biosafety of these LV. In addition to the removal of accessory genes that reduce or eliminate the pathogenicity of HIV-1, safer LV have been developed that reduce the probability of homologous recombination and mobilization of integrated vectors by splitting viral sequences on separate expression plasmids and deleting promoter and enhancer elements in the transfer vector itself. The intrinsic property of LV integration in the genome has even prompted researchers to develop an integrase-deficient LV.

Some recently developed HIV-1 derived LV have sufficient safety features included and can be used as gene transfer vectors for clinical trials. Moreover, while addressing concerns for the probability of RCL generation are important, it must be acknowledged that no RCL generation has been reported to date, neither during vector production nor during experimentation with laboratory animals. However, the further development of safer LV warrants continuous reconsideration of these risks and adequate containment measures.

The assignment of containment requirements cannot be generalized to all situations and therefore needs to be established on a case-by-case basis, in particular concerning transgene selection. This review provides recommendations

for the majority of commonly used research activities involving LV. We do however stress the importance of containment measures whose goal is to minimize direct exposure to LV and thereby prevent inadvertent transduction of the researcher.

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