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GUIDELINE ON DEVELOPMENT AND MANUFACTURE OF LENTIVIRAL VECTORS

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1. INTRODUCTION

Lentiviruses comprise a genus of the Retroviridae family (see footnote). They include the human pathogen Human Immunodeficiency Virus (HIV). Replication incompetent vector particles derived from lentiviruses have been shown to mediate transfer and expression of heterologous genes (transgenes) into a variety of cells. Dissimilar to other retroviral vectors, in particular those derived from gammaretroviruses (formerly known as oncoretroviruses), lentiviral vectors (LV) can mediate gene transfer into non-dividing cells, e.g. stem cells, lymphocytes, dendritic and nerve cells. Thus, in addition to use in *ex vivo* cell transduction LV could be useful for gene delivery *in vivo*. In addition, LV may allow for long-term transgene expression, as the transcript silencing observed with retroviral vectors (derived mainly from gammaretroviruses) is less frequent with LV and as such may provide the means for long-term *in vivo* clinical management of chronic diseases. However, in common with gammaretroviral vectors, LV suffer a number of drawbacks as gene transfer vectors, including (i) limited insert size (8 kb) of the transgene and regulatory sequences, (ii) difficulty in producing high titres of stable vector particles, and (iii) probability of activating or inactivating an endogenous DNA sequence that is localised near the proviral DNA integration site. Additionally, lentiviral genomes are more complex than those of the gammaretroviruses making design of LV a greater challenge.

Currently, in comparison with gammaretroviral vectors, gene transfer vectors derived from lentiviruses appear to raise greater quality, efficacy and safety concerns, especially since one of the main foci for development of LV has been their derivation from HIV, a severe human pathogen, although specific risks remain unknown. Major concerns regarding LV manufacture and clinical use are: (i) the potential generation of replication competent lentiviruses (RCL) during LV production, (ii) *in vivo* recombination with lentiviral polynucleotide sequences and (iii) insertional addition of proviral DNA in or close to active genes, which may trigger tumour initiation or promotion. Overall, the biohazards associated with the contamination of the LV with an RCL during production might be considered similar for all types of LV whilst the strategies for minimising such contaminations would be similar to those already in place for gammaretroviral vectors.

Many of the appropriate quality and safety requirements for retroviral vectors have been described in the current Note for Guidance (NfG) on the quality, preclinical and clinical aspects of gene transfer medicinal products, CPMP/BWP/3088/99). This guideline describes quality aspects and non-clinical testing that are in general relevant for LV that are intended for *ex vivo* or *in vivo* application. However, since there are wide differences in their genetics and design, a case-by-case assessment of quality aspects and non-clinical testing peculiar to individual LV types is not addressed herein; nor is this guideline intended to address specific efficacy and safety aspects of individual LV products.

It is recognised that the development and manufacture of lentiviral vectors is based on emerging technologies and that it is therefore expected there will be a need for ongoing revision of the guideline according to new scientific developments and any international harmonisation. Alternative approaches to those currently used to develop/manufacture lentiviral vectors may be taken provided they are appropriately detailed and justified.

Footnote: - Retroviruses are now classified into seven sub-genera: - alpha-, beta-, gamma-, delta-, and epsilon-retroviruses, plus **lentiviruses** and spumaviruses. **Gammaretroviruses,** formerly known as **oncoretroviruses** (e.g. murine leukaemia viruses {MuLV}, feline leukaemia virus {FeLV}, and gibbon ape leukaemia virus {GALV}), have been the most widely used category for vector development and have been available for clinical applications for up to 12 years.

2. NATURE OF PARENTAL LENTIVIRUSES AND IMPACT ON LENTIVIRAL VECTOR DEVELOPMENT

The human primate lentiviruses HIV-1 and HIV-2 are severe human pathogens, which target CD4+ T lymphocytes and macrophages. Other primate and non-primate lentiviruses are severe pathogens in their respective permissive hosts, but are, based on existing knowledge, not thought to be infectious/pathogenic in humans. Both the restricted cell specificity of HIV and the inability of non-

human lentiviruses to infect human cells may be overcome by replacement of the homologous viral envelope proteins by a gene encoding a heterologous viral envelope protein, conferring broad cell specificity. Although LV are designed to be replication-defective, there is concern about the potential generation of novel human pathogens from such vectors following clinical administration. However, for example, broad knowledge of HIV-induced pathogenicity has been generated and technical approaches to (i) design safe HIV-based LV and (ii) quantify HIV-based LV and detect potential recombination products are available. Nevertheless, perceived safety concerns (as above) on the clinical use of HIV-based LV have led to research and development of LV from suitable non-human primate- or non-primate-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 identified. The genetic organisation of the non-primate lentiviruses is simpler than that of HIV-1; EIAV is the simplest known lentivirus. EIAV and SIV vectors are at an advanced state of development. The consequences of human infection with LV derived from non-human primate- and non-primate-LV are unknown and thus safety concerns remain, particularly for the risks of horizontal and cross-species transmission of any mobilised, recombined chimaeric lentiviruses.

3. DESIGN OF LENTIVIRAL VECTORS

It is evident that all possible means should be employed to reduce any pathogenicity associated with the wild-type lentivirus used as the basis for LV production and to minimise risks associated with LV. This is achieved by: (1) generation of "minimal lentiviral genomes" through elimination of dispensible lentiviral virulence/accessory genes; (2) separation of lentiviral genes/sequences essential for LV generation into appropriate constructs/cassettes that reduce to a minimum the possibility of RCL generation. Currently most LV manufacturing processes employ at least three plasmids: an envelope construct expressing a heterologous viral envelope protein, e.g. the vesicular stomatitis virus glycoprotein (VSV-G), to replace the homologous lentiviral envelope protein and "pseudotype" LV particles; a helper construct encoding Gag and Pol viral proteins; a third construct harbouring the transgene, including the sequences needed for the production and packaging of active LV expression vector, commonly referred to as the transfer vector. Alternatively, LV is made using a construct encoding the transfer vector and a single cassette containing packaging and envelope genes, carefully designed to reduce the risk of recombination. In particular, sequence homologies between transfer and packaging (envelope and helper) constructs are minimised to prevent recombination. Concerning the transfer vector, modifications to alleviate the concerns for vector DNA mobilisation and proto-oncogene activation by promoter insertion in target cells is to be encouraged. For example, SIN (Self Inactivating) modification of HIV-based LV may prevent vector DNA mobilisation and recombination with wild-type HIV. On the other hand, some SIN vectors contain highly active promoters, such as the Cytomegalovirus (CMV) promoter, which contain enhancer activity that may result in proto-oncogene activation. Additional modifications to improve safety include adding genetic elements that transcriptionally partition helper components in a single packaging construct, separating *rev* in an independent construct, constructing synthetic *gag/pol* cassettes by exploiting favoured codon usage in human cells and replacing retroviral polyadenylation signal by exogenous ones. Such modifications are recommended providing these changes do not affect vector performance or introduce new safety risks. Comprehensive *in vitro* and/or *in vivo* experiments to assess construct characteristics including risk of RCL generation will be needed.

4. LENTIVIRAL VECTOR MANUFACTURING STRATEGIES

LV are produced either by transient co-transfection of a permissive cell line with a combination of packaging and transfer vector constructs or by transfection and culture of suitable packaging cell lines containing one or more incorporated (inducible) packaging constructs required for LV particle generation with the transfer vector. However, manufacturing by transient transfection limits the size of the production lot and thus may only provide sufficient quantities of LV for initial, limited clinical studies, most likely involving *ex vivo* transduction of cells. Production from stable packaging cell lines i.e. similar to those in existence for gammaretroviral vector production and of which several are now well-described, containing all necessary elements for LV propagation, might increase LV yields. The

requirements for the documentation of such cell lines, including full details of all incorporated sequences, and for their quality and safety as set out in the current NfG (CPMP/BWP/3088/99) should be followed.

Where manufacturing strategies utilise constructs that encode heterologous viral envelope proteins, e.g. VSV-G, for pseudotyping LV particles, contamination of LV lots by such envelope gene sequences should be removed in order to eliminate the risk of pseudotyping for endogenous or adventitious viruses. Contamination of LV lots with *gag/pol* sequences should be minimised, although they might not be completely eliminated as a result of non-specific co-packaging.

For the transient production by means of DNA co-transfection, construct DNAs to be used must be of high quality. The full sequence of the transfected plasmids and of the transfer vector packaged by the LV particles should be provided. The cell line used in manufacturing should be in compliance with the guideline on cell substrates (Note for Guidance on Quality of Biotechnological Products: Deriviation and Characterisation of Cell Substrates Used for Production of Biotechnological/Biological Products (CPMP/ICH/294/95)). Where applicable, batch-to-batch consistency of LV lots should be demonstrated (e.g., where sufficient accumulated analytical data from previous pilot batches used in clinical studies are available).

5. CHARACTERISATION AND CONTROL TESTING OF LENTIVIRAL VECTORS

Any preparation of LV should be fully characterised with regard to transducing activity, other characteristics relevant to vector particles and the absence of RCL. Lot-release specifications should be based on appropriate tests undertaken for characterisation (see NfG). For LV produced by transient transfection, the maximal level, if any, of contamination by plasmid DNA in the final lot should be set. DNase treatment to remove DNA may have to be considered. This treatment would be essential where DNA derived from constructs encoding VSV-G, other envelope proteins, or Gag/Pol, contaminate the LV product.

An in-house reference lot should be established from a fully characterised production lot.

5.1 Transducing activity

Important aspects of transducing activity are integration capacity, transgene expression and functionality. Transducing activity should be correlated with other relevant LV characteristics, such as particle numbers, Gag protein, Gag to envelope protein (or other protein) ratio and reverse transcriptase (RTase) activity.

Integration capacity

Integration capacity can be determined by demonstrating the incorporation of vector proviral DNA into target cells; it can be evaluated using limiting dilution techniques of transduced cells and NAT assay with probes targeted to the transgene, or to the packaging signal ψ, contained within the transfer vector DNA.

Such an assay requires standardised procedures, including specification of (1) LV titres for infection, (2) the cell line used for transduction, (3) cell culture conditions for transduction, (4) timing of PCR testing after transduction (5) quantitative estimation of the number of transduced cells and (6) a pseudotype-specific lentivirus reference reagent. Most LV pseudotyped with VSV-G infect a wide range of cell lines. It is recognised that cell lines widely used and characterised for the detection of pseudotyped retroviruses, including lentiviruses, might be of variable quality due to differing culturing conditions in different laboratories. This may affect standardisation of assays and thus consistency of results.

Transgene functionality

Quantification of LV-related transgene functionality is typically performed by measuring the concentration and functional activity of the expressed transgene product. Such assays require

standardised procedures, including specification of (1) LV titres for infection, (2) the cell line used for transduction, (3) cell culture conditions for transduction, (4) timing of transgene product expression and other considerations as included under "Integration capacity".

5.2 Lentiviral Vector particle quantification

Other than transducing activity, quantitative measures of LV particles to demonstrate consistency of LV lots should be made. A routine method for detection of retrovirions is negative staining electron microscopy, however, quantification of virion particles by this technique is difficult (due to particle plasticity and instability) and insensitive. Alternatively, total particle number can be estimated by immunostaining of viral envelope or capsid proteins and visualising these by con-focal microscopy in relation to a known concentration of fluorescent microspheres. However, as formation of incomplete LV particles lacking the envelope protein occurs, estimation of the proportion of incomplete particles could, in certain instances, be of value although it is recognised that at present such estimations remain technically challenging. Typically, for lentiviruses, 1 pg Gag is approximately equivalent to 10^4 particles. However, in some lentiviral expression methods, Gag can be over-expressed and empty LV particles devoid of transducing activity generated. Nevertheless, it could be useful to provide data correlating Gag protein and transducing activity for each LV preparation.

Additionally, the level of RTase activity may be usefully correlated with the number of particles and transduction efficiency. Measurement of RTase activity, as well as measurement of the Gag protein, is an indirect method for analysing LV particle number. It is recognised that such determinations are not in themselves sufficient for LV lot characterisation.

To estimate the numbers of LV particles with vector RNA molecules, validated quantitative nucleic acid amplification technology (NAT) based assays, e.g. real time polymerase chain reaction (PCR) technique, may be used for comparison with transducing activity. However, since DNA contaminants, e.g., from transient transfections and co-packaged *gag/pol* sequences may be present in LV lots, appropriate precautions should be implemented to minimise interference by such contaminants in NAT based assays.

5.3 Testing for Replication Competent Lentiviruses

Despite current safeguards in LV production systems to eliminate the possibility of RCL generation, there remains a low risk of them contaminating LV lots and therefore appropriate tests should be applied to test for their presence.

The presence of RCL can be determined by several means. For example, following infection of a susceptible cell line with RCL and serial passaging of successive cell supernatants to achieve RCL amplification, quantitative real-time PCR can be used to detect integrated *gag/pol* specific nucleic acids. For instance, for HIV-1-based LV manufacture, any RCLs generated would only share the *gag* and *pol* genes with HIV-1. Detection of such RCLs could be based on HIV-1 Gag protein or *gag* gene sequences, for which there are well-validated and sensitive assays already available, e.g. p24 Gag immunocapture assay or *gag* RNA PCR assay, respectively. In addition to Gag/Pol, RCLs would express the vector envelope protein, e.g. VSV-G. Therefore, where VSV-G is the pseudotyping envelope protein, it is also appropriate to use a VSV-G immunoassay and/or molecular assay for *VSV-G* DNA or RNA to detect RCL replication. Alternatively quantitative RTase assays, following RCL amplification involving several serial passages of a susceptible cell line, should be considered.

As a further alternative, "marker rescue" assays, in which a specific marker gene is rescued by RCL, could also be considered for their detection.

In general, the limit of quantification required for RCL tests should be established according to the proposed LV dose and to the size of the production lot. Ideally, the capacity of tests to detect one RCL in a vector dose should be proven. However, the choice of a suitable, representative positive control or reference standard is critical for demonstrating the sensitivity of RCL assays.

Any RCL assay should be well characterised and include an appropriate positive control. However, for tests requiring cell infection the current lack of generic reference materials poses a challenge for assay

calibration. Presently, it is considered undesirable to generate a lentivirus encoding a heterologous Env (e.g.,VSV-G) and Gag/ Pol simply to monitor RCL infectivity assays, since such a virus could be pathogenic. For HIV-based LV, an attenuated HIV lacking all accessory genes as a positive control for RCL assays could be of value.

6. ONCOGENESIS

Due to the wide distribution of proviral DNA insertion sites, the risk of oncogenesis due to insertional mutagenesis increases with the number of cells modified and with the number of insertions per cellular genome. While the integration capacity of LV lots may be assessed in suitable cell lines by NAT methods, identification of the sites in the human genome at which vector DNA integration occurs and their consequence may contribute to safety assurance. For such investigations in the case of *ex vivo* transduction, archiving a small fraction of the transduced cells for clinical use should be considered.