National Institutes of Health
Gene Therapy Policy Conference

LENTIVIRAL VECTORS FOR GENE DELIVERY
March 9, 1998

INTRODUCTION

The primary goal of any gene therapy is to achieve successful gene transfer and expression in a target cell. While some laboratories continue to explore the potential of gene therapy in the treatment of genetic diseases, others are applying this technology to diverse diseases such as cardiovascular disease, cancer and AIDS. Ultimately, for gene therapy to be successful the genetic (therapeutic) material (e.g. DNA) must be efficiently transfected (or transduced) and its expression sustained in the target cell. There are two general categories of delivery vehicles/vectors employed in gene therapy protocols. The first category includes the non-viral vectors, ranging from direct injection of DNA to complexing the DNA with polylysine, cationic lipids and/or cell-receptor ligand. The second category is comprised of DNA and RNA viral vectors.

Viruses have evolved specific mechanisms to deliver their genetic material to target cell nuclei. The study of these mechanisms led to the concept of using viruses as agents for gene transfer, and progress in recombinant DNA technology has made this concept a reality. The current recombinant viral vector designs involve incorporation of the foreign (therapeutic) gene sequences into the genome of a parent replication-defective virus. Virus members of the family *Retroviridae*, e.g. retroviruses and lentiviruses, are among the most widely used viral vectors. Retroviruses have many desirable attributes making them particularly useful as gene delivery vectors, such as i) a well understood replication cycle, ii) an ability to express foreign genes upon infection of many cell types, in vitro and in vivo and iii) the capability of integration (as proviral DNA) into the target cell genome permitting passage of the therapeutic gene to cell progeny. Infection and integration of target cells by retroviruses is limited to dividing cells. Lentiviruses have an advantage over retroviruses in that they can infect both dividing and non-dividing cells and therefore have recently attracted much attention regarding their potential as vectors for gene delivery/therapy.

The following consensus document summarizes the lentivirus vector issues as presented at the National Institutes of Health, Gene Therapy Policy Conference on "Lentiviral Vectors for Gene Delivery," March 9, 1998.

THE BIOLOGY OF PRIMATE LENTIVIRAL INFECTIONS
Malcolm Martin

HIV was demonstrated as the etiologic agent of AIDS about 15 years ago and has since been shown to be a lentivirus. Lentivirus can infect hematopoetic cells and cause persistent infection but has yet to be found in germ line cells. They typically present extensive genetic and antigenic variation. One unique feature of the lentivirus life cycle

is the integration of its proviral DNA into the host genome which is a desirable property for gene vectors. The HIV genome is well characterized. The LTR promoter binds transcription factors whish are required for efficient expression of the viral genes and is required for viral genome integration. The tat gene product mediates efficient RNA expression from the LTR. Rev is involved in RNA splicing and transport from the nucleus to the cytoplasm. Additional accessory proteins include vif, vpr, vpu and nef. Vif is present in small amounts in the mature virion and functions early in infection. Vpr is a karyophilic protein involved in transportation of the pre initiation complex to the nucleus of infected cells. It is also capable of arresting cells in G2 phase. Vpu has ion channel activity and facilitates the release of progeny and degradation of CD4 receptor in infected cells. Nef causes down-regulation of CD4 expression and enhances HIV infectivity in peripheral blood lymphocytes. The precursor protein gag is processed into 4 proteins which form the structural frame work of the HIV virion. Another precursor protein gag/pol is processed into the 3 proteins protease, integrase and reverse transcriptase. The protease is involved in virion maturation through protein processing. Integrase catalyzes the integration of the proviral DNA into host cell DNA.

Reverse transcriptase converts viral (genome) RNA into proviral DNA. The envelope gene product is synthesized as a precursor (gp160) and is cleaved into gp120 and gp41. Their major function is to bind to CD4 receptor on lymphocytes. The wide antigenic variability observed for HIV resides in the gp120 and this can result in as much as a 50% variability among different isolates. Presently it is still unclear what factors (host or viral) are responsible for sustaining long-term infection and are involved in the destruction of the immune system.

LENTIVIRAL VECTORS FOR GENE DELIVERY
Inder Verma

Retroviral and adenoviral based vectors have been used for gene delivery. Retroviral vectors have limitations in their use in that they are unable to infect post-mitotic cells due to a pre-initiation complex (PIC) which is unable to enter the nucleus and integrate in a non-dividing cell. Adenovirus vectors are limited by their induction of both a humoral and cellular immune response to viral proteins, especially upon repeated administration. Lentiviruses are good candidates for gene therapy because their PIC has karyophilic properties responsible for entry and integration into the nucleus of nondividing cells.

Recombinant replication defective lentiviral gene delivery vectors were constructed using a three vector system. These consisted of a packaging construct, a transgene vector and an envelope construct. First generation packaging vectors lacking a LTR contained all viral genes (except envelope) and were expressed under the control of a heterologous viral [Cytomegalovirus immediateearly (CMVIE)] promoter. The transfer vector contained flanking LTRs, packaging signal sequence and a transgene whose expression is under control of a heterologous promoter. The third vector expresses a non-HIV envelope protein, such as vesicular stomatitis virus g (VSV-G) protein. The use of VSV-g expands the target cell tropism of the vector and aid in reducing the probability

of producing RCR (replication competent recombinant) lentivirus (HIV).

Unlike MLV based vectors, lentivirus vectors can infect and express their transgene in nondividing cells in vitro as well as efficiently transduce monocyte derived macrophages. Integration is required for functional transduction since expression is absent in vectors containing a mutation in their intergrase gene. These vectors can be used to deliver transgenes directly into a variety of nondividing cell types in vivo. These cell types include post-mitotic neurons of the brain, muscle myocytes, liver cells and retinal epithelial cells. In post mitotic neurons transgene expression is very high (80-90% of neuronal cells at the site of gene delivery) and still observed at one year post delivery. In order to increase the safety of the in vivo application of the lentivirus vector - vector (plasmid) construction was further modified to include deletions in accessory proteins and in specific regions of the 3' U3 TR region not involved in integration.

To determine whether lentivirus vectors can function in gene replacement therapy, a mouse gene-knock out model for clotting factor IX was developed. Adenovirus vectors elicited induction of anti-factor IX antibodies. Lentivirus vectors have yet to be tested in this system.

MULTIPLY ATTENUATED LENTIVIRAL VECTORS: SAFETY AND PERFORMANCE
Didier Trono, M.D.

Lentiviruses are unusual among retroviruses in their ability to infect non-dividing cells. For HIV-1, this property reflects the recognition of at least three components of the preintegration complex by the cell nuclear transport machinery: matrix (MA), Vpr and integrase (IN). MA and IN mediate HIV nuclear import through their ability to bind the members of the importin/karyopherin-alpha family. Mutation of the intergrase prevents nuclear localization. Transportation into the nucleus is a prerequisite for integration and expression. There are two pathways for nuclear localization of the pre-initiation complex. The first is MA/IN directed localization via their NLS and interaction with karyophilic alpha. The second pathway involves Vpr, which does not carry a prototypic nuclear localization signal (NLS). It seems to function as an importin/karyopherin-beta analogue which enhances MA- and IN-mediated nuclear import. This apparent functional redundancy may be due to cell-specific differences in the utilization of the various mediators of HIV nuclear import. To decrease the probability of generating replication competent recombinant (RCR) virus a second generation vector was created which is deleted in its accessory genes vpr, vpu, vif, nef . These represent crucial virulence factors of HIV. These deletions did not affect their performance either in vitro or in vivo. In order to further improve their safety a third generation of lentiviral vectors was developed in which 1) the transgene vector contains a deletion in the 3' LTR U3 region making it self-inactivating in target cells and 2) the packaging vector is a split genome construct with the tat gene deleted and the rev gene expressed by a separate non-overlapping construct. This vector achieves transduction of target cells as well as that observed with the previousgeneration.

HIV's ability to infect nonmitotic cells can be exploited to create retroviral vectors that

allow for the in vivo delivery, integration, and sustained long-term expression of transgenes into non mitotic cells such as neurons, hepatocytes and pancreatic islet cells. Vpr appears to be completely dispensable for transducing efficiently all of these tissues. In addition, the Vif, Vpu, Env, Nef and Tat proteins, which all represent crucial virulence factors of HIV-1, can be deleted from the vector system without affecting its performance. The new generation of HIV vectors will thus include only Gag and Pol as its HIV-derived packaging components. This should greatly facilitate envisioning its use for human gene therapy.

REGULATED DETERMINANTS OF LENTIVIRAL ENTRY
Garry P. Nolan, Ph.D.

Post HIV-1 entry, productive HIV-1 infection of primary T cells require overcoming blockades at one of several steps. For example, quiescent T cells allow uptake of HIV but there is no reverse transcriptase activity or no nuclear transport of the viral genome. Overcoming this block is dependent upon host intracellular events that are induced concomitant with T cell activation. These events include activation of transcription factors such as NFkB and NFAT. NFATc/NFAT2 is a sufficient cellular factor for engendering productive HIV-1 infection in primary CD4+ T cells. Ectopic expression of NFATc in primary T cells-- in which T tropic HIV-1 are normally blocked at 1st strand transfer and does not complete productive infection-- is sufficient to allow completion of reverse transcription as well as permit productive HIV-1 infection. Thus, the mechanism of HIV-1 replication of primary T cells can be controlled by the regulation of host permissivity factors such as NFATc. Such permissivity factors, or other host factors they might regulate, represent challenges to the use of certain classes of lentiviral vectors in some cell types that maintain such blockades. Feline Immunodeficiency Virus (FIV) based retroviral packaging systems to address safety concerns relating to HIV-1 based packaging systems (ie, mobilization or recombination of HIV-1 based vectors in individuals who are concurrently HIV-1 positive or become HIV-1 infected post delivery of HIV-1 based vectors). Packaging cell lines are being developed for improved vector production. The FIV vectors are capable of infecting nondividing cells.

HUMAN AND NON-HUMAN LENTIVIRAL VECTORS FOR GENE DELIVERY
Alan Kingsman, Ph.D.

Retroviral vectors are attractive vehicles for therapeutic gene delivery for a variety of reasons. However, one major disadvantage of the oncoretrovirus-based vectors is their dependence on mitosis for transduction. Since the discovery that lentiviruses could infect certain non-dividing cells this subgroup has been the focus of the development of a new type of retroviral vector that is able to transduce non-dividing cells. Recently, a minimal vector system that lacks the HIV-1 based vectors, were designed to be produced from transient three-plasmid transfection. The packaging vector contained the gag-pol but lacks all of the viral accessory genes except for Rev/RRE. The vector was pseudotyped with VSVg envelope. These vectorsefficiently transduced aphidocolin treated (nondividing) cells.

Although the minimal HIV vectors have an improved safety profile, it is conceivable that they will not be used for conditions other than HIV infection and AIDS. Therefore, a vector systems based on the non-primate EIAV was developed. EIAV are structurally simpler than HIV. They have genes for gag, pol, rev, tat and S2 but lack the accessory genes found in HIV. First generation vectors were derived from a three plasmid transfection system. EIAV based VSVg pseudotyped viral vectors were generated with all the viral genes and resulting vectors produced equivalent titers (measure by marker gene transduction) in dividing and non-dividing cells. The next generation of vectors was produced after further elimination of viral cis sequences such as packaging elements. These vectors were also capable of transducing a range non-dividing cells, in culture and *in vivo*, with efficiencies comparable to HIV vectors. These virus vectors can be concentrated to achieve titers up to 10(7). RCR assays after multiple passage of transduced cells showed three generations of helper virus. Injection of these vectors into the caudate nucleus of the rat brain demonstrated effective gene transfer into neurons. Retrograde transport to the thalamus was also observed.

The issue was also raised whether slow dividing cells (e.g., glial cells and hypoxic regions of solid tumors) also could be targets for lentiviral gene therapy. The latter generation EIAV vector was capable of transducing a breast tumor spheroid as measured by transgene expression which suggests these vectors may be applicable for tumor gene therapy.

EQUINE LENTIVIRUS VECTORS
John C. Olsen, Ph.D.

Equine infectious anemia virus (EIAV) is a member of the lentiviral group of retroviruses. Though it causes disease in horses, EIAV replication is highly species-specific, and there are no known cases of human infection. EIAV possesses several features that may be attractive for a gene therapy vector, including the ability to replicate to high titer and the ability to efficiently integrate its DNA into chromosomal DNA of non-dividing and terminally differentiated cells.

Replication-defective EIAV vectors encoding a transgene (e.g., puromycin-N-acetyl transferase or ß-galactosidase) were derived from either a three plasmid transfection system or a two plasmid transfection system in a stable cell line which expresses EIAV viral proteins. In transfection studies it was found that vectors can be pseudotyped with envelope glycoprotein G from vesicular stomatitis virus (VSV). These vectors can transduce the transgene into cultured human fibroblasts, at a transduction rate of 50%. Transcription was only observed from the internal (transgene) promoter and not the LTR. Transgene expression was also observed after transduction of aphidocolin treated (i.e., non-dividing) cells. MuLV based vector could not transduce these cells.

HIGH-TITER HIV-1-BASED VECTOR SYSTEMS
Jakob Reiser, Ph.D.

Previously designed novel pseudotyped high-titer replication defective HIV-1 vectors

were based on a two plasmid packaging system whereby one plasmid contained HIV backbone with a deletion in the envelope gene. The transgene under heterologous promoter control, was positioned within the HIV backbone. Pseudotyping of the vector was provided by expression from a second plasmid expressing a heterologous envelope protein such as VSVG or MuMLV-A-env. High titers (10(7)) of pseudotyped HIV-1 particles were achieved. In order to improve on the safety and retain the flexibility and efficiency of the vector system, pseudotyped HIV-1 particles were generated by transient transfection of human embryonic kidney 293T cells with a defective packaging construct, a plasmid encoding a heterologous envelope (Env) protein, and a vector construct harboring a reporter gene. The packaging constructs lack functional Vif, Vpr and Vpu proteins and/or a large portion of the Env coding region as well as the 5 and 3 long terminal repeats, the Nef function and the presumed packaging signal. Titers of the first and second generation of these vectors were comparable. Titers were adversely affected when tat was deleted suggesting that a functional tat, in this system, should be retained. Packaging constructs with a mutation within the integrase (IN) core domain profoundly affected colony formation and expression of the reporter gene, indicating that a functional IN protein is required for efficient stable transduction. Comparison of vectors created with single or multiple deletions/mutations in the accessory proteins were not significantly different in their ability to transduce target cells.

Other env proteins have been explored regarding their capacity to allow the formation of pseudotyped HIV-1 particles such as rabies virus and Mokola virus G proteins which yielded functional pseudotypes. Improved vector system successfully transduced lacZ (transgene) into cardiac miocytes suggesting accessory proteins are not required *in vivo* in these vectors. Serial passage (6-7 weeks) of H9 T cells infected with these vectors showed a decrease in p24 levels to background as determined by ELISA. This suggests there is no *de novo* generation of replicating virus.

DELIVERY OF LENTIVIRUS CFTR INTO THE LUNGS
James M. Wilson, M.D., Ph.D.

A replication defective vector based on HIV was evaluated for gene transfer directed to lung air way epithelial cells. The tropism of this vector has been expanded through the incorporation of the vesticular stomatitis virus G protein into its envelope. The HIV vector effectively transduced non-dividing airway epithelial cells *in vitro* whereas a murine based retroviral vector did not. Experiments in a human bronchial xenograft model in nude mice (lack immunity for graft rejection) demonstrated high level gene transduction with a CFTR (cystic fibrosis transductance receptor) containing HIV vector into undifferentiated, cystic fibrosis (CF) - derived cells of the xenograft. After the graft matured, CFTR expression was stable and capable of functional correction of the CF defect, as measured by channel activation from chloride ion transport and restoration of epithelium to resist bacterial infection. The HIV vector did not effectively transduce cells of the xenograft when instilled after the epithelium had differentiated. Although post-entry restrictions cannot be ruled out, this block to transduction appears to be at the level of entry although post entry since no viral DNA was detected by sensitive PCR assays in the transduced well-differentiated cells. Differentiation of airway epithelial cells

polarizes receptors thereby preventing viral infection. This suggests a need to design novel pseudotyped vectors to overcome this problem. Further development of this vector system for CF gene therapy should focus on a better understanding of potential entry and post entry blocks.

LESSONS FROM HIV VACCINE DEVELOPMENT
Michael S. Wyand

Live attenuated SIV may serve as a model for the development of potential vaccines against AIDS. Lessons learned from the development of an AIDS vaccine may or may not be directly applicable to the development of lentivirus gene delivery vectors since the viral genes responsible for pathogenesis may be eliminated from the delivery vectors. One attenuated SIV, SIVmac239delta3 is deleted in vpr nef and NRE was compared to other SIVmac deletion mutants for their level of viral attenuation. Delta3 was more attentuated than single virus mutants in any of the three genes. Complete attentuation of the virus was achieved with a delta5 virus containing mutation in nef, vpr, vpx, vif and ltr. For these virus mutants, reduction in virus replication correlates with decrease in pbmc (peripheral blood monocytes) viral load - that is, virus recovery from these cells, anti-SIV antibody response and protection SIV challenge.

Safety issues regarding the development of an AIDS vaccine were discussed. First, in adults the delta3 virus only virulent in 5% of the target population whereas virus with additional deletions did not cause disease and neonates are a more sensitive population. Another issue is how long post-infection should one continue to follow animals for potential virulence. Additional issues include mucosal transmission, germ line establishment of the viral genome, exposure of vaccine to immuncompromised individuals, insertional mutagenises or other oncogenic potential and recombination. Animals chronically infected with deletion mutant viruses show no viral reactivation, however, endogenous recombination has been observed between two different single mutant viruses. This suggests that administration of single deletion mutants vaccines of differing types should be avoided. It was re-emphasized that lentiviral delivery vectors will be safer than vaccine vectors due to the removal of many of the viral genes.

DEVELOPMENT OF SAFE AND EFFECTIVE LENTIVIRAL VECTORS
L. Naldini, M.D., Ph.D.

Lentiviral vectors combine high efficiency of integration with the transduction of non dividing cells. They are being evaluated for *ex vivo* as well as *in vivo* gene delivery. A first generation hybrid vector was derived in part from the HIV-1 virus, and pseudotyped by the envelope of the VSV or of the amphotropic MLV. The vector transfers an expression cassette for the transgene flanked by *cis*-acting sequence of HIV-1 without any viral gene. The use of a heterologous envelope broadens the tissue tropism of the vector, and makes impossible the generation of wild-type virus during vector production. These vectors transduce dividing and nondividing cells (*in vitro* and *in vivo*). For clinical applications, lentiviral vectors must comply to strict safety standards, given the nature of the parental viruses. For instance, they must be replication defective and not capable of

transferring viral genes to target cells. Strategies to ensure biosafety of lentiviral vectors involve: (i) the design of packaging constructs which make recombination with transfer vector unlikely and multiple events required for productive combination, (ii) separate expression construct for viral genes, (iii) use of minimal set of HIV sequences especially those which have pathogenic properties. A minimal set of HIV sequences necessary for efficient transduction was defined. As the packaging and the transfer vector constructs were improved, new "generations" of the vector system were developed in order to comply with clinical applications and biosafety issues. A second generation vector system included a minimal packaging construct with deletion of all accessory genes (vif, vpr, vpu and nef). A third generation vector system includes: (i)a split-genome minimal packaging constructs with the *tat* gene deleted and the *rev* gene expressed by a separate non overlapping construct and (ii) a5' chimeric self-inactivating (SIN) transfer vector with the enhancer/promoter in the U3 region of the 5' and 3' LTR respectively replaced and deleted.

Vectors of the new generations were as efficient as those derived from wild-type construct at delivering transgenes *in vitro*, and *in vivo* into neurons. The deletion of most LTR sequences and of up to six genes essential for HIV pathogenesis eliminates the possibility that even the unlikely replication-competent recombinant (RCR) arising during vector production would have any of the pathogenic features of HIV. There was no evidence for lentivirus vector/HIV recombination in SupT1 cells (endogenous HIV) exposed to lentivirus vectors (all generations). As the only features of the parental virus shared by any recombinant would be those dependent on the *gag* and *pol* genes, RCR monitoring can be performed by such sensitive assays as HIV-1 Gag p24 immunocapture or HIV-1 *gag*RNA PCR. Of note, any RCR would be sensitive to anti-HIV drugs that target reverse transcriptase or the viral protease.

BIOLOGY OF LENTIVIRUS INFECTION IN NON-HUMAN PRIMATES
R. Paul Johnson

The study of non-human primate lentivirus biology may lead to a better understanding of potential safety issue regarding the usage of lentiviral vectors in humans. Primate lentiviruses are nonpathogenic in their natural host. This is exemplified by African green monkeys infected with SIVagm where these animals have been infected for greater than 10,000 years with no significant effect on the species. Cross species transmission is primarily responsible for primate lentivirus pathogenicity. HIV-2 is believed to be derived from SIVssm (sooty mangabey monkey) through infection from sooty mangabey monkeys. It is hypothesized that HIV-1 may have originated from a primate reservoir. Nonhuman primates are not good models for the study of HIV pathogenisis due to low level of virus replication and general lack of AIDS development in these hosts. Chimeric HIV/SIV viruses were constructed several of which could replicate both in monkey and human cell lines. The study of chimeric HIV2/SIV based lentivirus vectors in macaques may provide a means of analyzing the immunogenicity and pathogenicity of HIV2/SIV in primates.

PRECLINICAL SAFETY EVALUATION OF LENTIVIRUS VECTORS: FDA

REGULATORY EXPECTATIONS
Anne M. Pilaro, Ph.D.

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In developing novel products for use as vectors in gene therapy, short-term animal studies are typically required to demonstrate the safety of these agents prior to their introduction into humans. The goals of these studies are to obtain information regarding a safe starting dose and escalation scheme for the clinical trial, to identify target organs of toxicity and parameters to monitor in the clinic, and to identify any significant issues which may pose an unacceptable risk to the patient population being treated. Therapies employing viral or plasmid DNA vectors as novel delivery systems present additional, unique issues regarding determination of safety, which require preclinical study designs targeted to answer specific questions. Depending on the route, frequency and duration of exposure, as well as the type of vector employed, animal models may be designed to evaluate specific concerns regarding the safety of gene transfer. In general, the issues involved in designing and developing appropriate preclinical testing to determine the safety of recombinant lentiviruses as vectors for gene therapy will be similar to those encountered for other viral or plasmid DNA vectors.

However, specific concerns regarding the potential for vector recombination, trafficking to non-target tissues after injection, and interactions of the host immune system with the transduced cell population should also be considered in designing these studies. Because of these concerns, the Agency encourages sponsors to perform toxicology testing of lentiviral vectors planned for use in gene therapies in a species relevant to their clinical model. Early identification of the safety concerns anticipated in the clinical trial can result in preclinical data which facilitate entry of these novel vectors into humans and ensure their safety throughout the course of product development.

BIOSAFETY ISSUES IN THE CLINICAL APPLICATION OF LENTIVIRAL VECTORS
Dale G. Ando, M.D.

There are several biosafety issues that need to be considered in the clinical application of lentiviral vectors. There are several potential problems that may occur with the uses of lentiviral vectors on HIV patients. There is a increased potential for RCR and vector mobilization and simple assays for their detection are not available. An additional problem may be the requirement to discontinue anti-HIV therapy prior to *in vivo* gene transfer with lentivectors. Mobilization of lentiviral vectors by HIV would require their co-expression within the same cell. Lentiviral vectors which include envelope pseudotyping and/or are self inactivating vectors may limit their mobilization. If mobilization of the lentivirus vector occurs, infection can be treated with retroviral chemotherapy. These issues demonstrate the need to develop preclinical models for lentiviral mobilization and to assess their potential risk. Insertional mutagensis and germ line transfer also present additional risks. However, increased rates of cancer due to insertional mutagenesis have not been observed in HIV infections nor with retroviral gene therapy. Increased rates of birth defects due to germ line integration due to germ line integration have not

been seen in HIV infections. However, envelope pseudotyping alters lentiviral host tissue range and could increase the risk of germline insertion. Lentiviral replication competent recombinants may arise from: (i) producer cell lines during their manufacturing, (ii) *in vivo* gene transfer, and (iii) *in vivo* infection with HIV. RCR monitoring in HIV negative patients using an HIV-RNA (gag) PCR assay. Testing for a replication competent and mobilized virus in HIV patients cannot use simple PCR techniques and will require isolation and characterization of the virus. The incremental risk strategy for the development of new viral delivery systems may be based on the target population: (i) *ex vivo* gene transfer in cancer patients, (ii) *in vivo* gene transfer in patients with fatal or high morbidity disease, and (iii) *in vivo* gene transfer in chronic disease patients. Risk/benefit assessment may be based on fatal versus chronic disease patients, potential for efficacy and availability of alternative treatments, site of therapeutic gene expression, mode of vector delivery (*ex vivo* vs. *in vivo* gene transfer) and patient age and reproductive status.

FIRST STEPS TOWARD COMMERCIAL USE OF LENTIVIRUS VECTORS
Douglas J. Jolly, Ph.D.

Lentiviral vectors are being developed to exploit the potential that they have for transducing tissues that are not easily transduced by vectors based on murine leukemia virus. In addition, it seems possible that preparations from the lentiviral system may be intrinsically more potent. A murine retrovirus-based anti-HIV immunotherapeutic vector (HIV-IT) was generated to express HIV rev and envelope proteins in order to induce/augment an anti-HIV CTL response. *In vivo* efficacy trials revealed no difference between placebo and HIV-IT treated groups, possibly due to patients undergoing alternate anti-HIV therapies. However, the trial did reveal that there were no adverse effects, such as no difference in onset of HIV associated disease, resulting from extended HIV-IT treatment. No RCR nor germ line transfer was detected. Three to four single course injections did not induce an anti-vector immune response. However, in some patients receiving nine to twelve injections, an anti-vector immune response was elicited but may be attributed to the large burden of foreign material injected into the patient.

For any commercially developed vector to target for human trials, animal data should first be obtained to establish biological activity and safety. For human trials dire disease should be targeted for first use to establish safety but eventually expanded to target less severe disease.

Session I: Lentivirus Biology and Vectors
Roundtable Discussion

Chair:
Peter K. Vogt, Ph.D.

Panelists:
Alan Kingsman, Ph.D.

Malcolm Martin, Ph.D.
Garry P. Nolan, Ph.D.
John C. Olsen, Ph D.
Jakob Reiser, Ph.D.
Didier Trono, M.D.
Inder M. Verma, Ph.D.
James M. Wilson, M.D., Ph.D.

Jolly: Does Dr. Wilson's xenograft model produce mucous, and might this be an explanation for decreased transduction in more well differentiated cells?

Wilson: Yes, this may be a mechanical barrier to vector binding to cells.

Audience: Two pathways for lentiviruses were proposed. Our work suggests that a matrix may serve as a bridge between the preintegration complex, but experiments by others suggest that its function may not be required for transduction. This may be a tissue specific phenomenon. In the second generation vector, the accessory factors appear to be responsible for only a 2- to 3-fold difference, but they may be important *in vivo*.

Trono: We see no difference in the *in vivo* models, although hematopoietic stem cells systems have not been investigated. VPR seems to be the only accessory gene that is helpful.

Verma: We cannot tell which of these accessory factors are necessary, but VPR and VPU minus preparations transduction of liver do not seem to make a difference.

Trono: There is a report that Tat might only promote transcription. Experiments suggesting that with producer cells suggesting that transduction was dependent upon Tat were complicated by potentially-confounding technical factors. Its function in transcription and is not necessary.

Martin: The 5-prime UTR region can be made independent of the Tat region. It is clear that nuclear translocation is required and must be efficient to infect cells.

Verma: There may be nuclear factors.

Audience: What are the drawbacks of designing the next generation? Wild type HIV may integrate non-randomly, and might it be possible to design a next generation that could accomplish site-specific integration?

Verma: There is no difference in the quasi-non-random integration of HIV compared to other retroviral vectors. The problem remains the same as for MLV vectors.

Trono: Fusion of integrase to other factors will be important in targeting to specific sites of plasmid DNA, but may not work on the intact chromosome *in vivo*, so such targeting

strategies may require additional research.

Verma: What we do not know is a large amount of DNA which is not in the ideal double-stranded form that is capable of integration, but the amount is unknown.

Wilson: Integrated and non-integrated DNA are not easily differentiated. It is impossible to know which form might be responsible for expression.

Naldini: We have tried to isolate integrated and non-integrated DNA. We do see some level of expression from non-integrated. This does not appear to be responsible for long-term expression and is a relatively small amount of the expression.

Martin: Arrested lymphocytes in G0 are uninfectable with HIV. In my opinion, reverse transcription occurs, but this is not integrated. The results shown by Jim Wilson are compatible with this explanation. If DNA is unintegrated, it is not stable and is lost. With integrase mutations, there may be an increased rate of DNA synthesis. But without integration, the presence of large quantities of unintegrated DNA may produce a large amount of RNA, and this may be expressed.

Trono: G1 or G2 arrested cells may be different from arrested T-cells and fundamentally different. The energy level in other cells such as the resting liver cells may be different, and this may be necessary for the importing thus accounting for efficient transduction. If the preintegration complex is properly docked at the nucleopore, subsequent activation may result in integration.

Verma: The preintegration complex may be more stable.

Vogt: This brings up the question of insertional mutagenesis.

Verma: Long-term problems beginning with MLV virus. The long-standing work of French Anderson and colleagues is probably the best experience on this issue, and calculations of the number of viral particles administered to patient cells, and the lack of problems is the result thus far.

Verma: HIV may kill a large number of cells, and multiple integration may not be at issue in this case.

Trono: There is no evidence that any of the tumors associated with AIDS are due to HIV integration and mutagenesis.

Audience: Regarding a high-molecular weight band on one of the illustrations presented by Dr. Wilson, would this represent lentiviral integration?

Wilson: This would appear to be an artifact of incomplete restriction digestion. Our titer of 107 was attained early in our experience, but not typical of our experience.

Naldini: The best concentration of vector is 107/ml. Transient transfection is optimized on 293T cells, which can be concentrated 3-log-fold. This is under optimized, careful conditions.

Martin: What are the marker genes used for titering? What fraction represents non-integrated?

Naldini: Several markers have been used. With integrase, we get a 2-log different from wild-type. Most expression from this is integrated. Integrase mutants yield some integrated material.

Audience: Did you try other arrested cells?

Naldini: We did not do those experiments.

Audience: Could you share with us why you became interested in FIV vectors; why go with animal viruses, and why FIV? And what are the requirements for VIF and ORF2?

Nolan: There is no need for VIF. The rationale to go to an animal virus was based largely upon the response of the IRB and the IBC at Stanford and were highly restrictive. No evidence of sero-conversion suggested a host-restriction; that would imply that it would be more safe, and allow us to begin experimentation.

Audience: Does HIV vector in HIV-infected patients result in mobilization *in vivo*?

McIvor: Why use a human lentivirus when non-human vectors may be just as effective? Why should it make a difference?

Verma: The answer is in your question: why should it make a difference.

Trono: The past 20 years of experience, including people who have been infected with deleted vectors, and who have not been infected.

Nolan: There is the un-suspected pathogenicity of non-primate vectors that may be most concerning.

Martin: These viruses have been around for millions of years. Take a simple process such as reverse transcription; these are not chemical reactions occurring in an isolated test tube. They have been moving around in the genome. The human HIV has evolved to work well in the human cell. People have used simian lentiviruses; SIV required the simian GAG and POL to produce SIV in monkey cells.

Kingsman: As far as we can tell, these proteins are functioning in the same way. In a reasonable world there probably would be no problem. However, it is the public perception that will lead to use of non-primate systems.

Trono: HIV has been documented in the human population for, perhaps, 80 years and attenuated virus have also been demonstrated. No such experience has been accumulated with other lentiviruses (so this is an important experience with HIV to be exploited).

Audience: What is the possibility of using HIV vectors in naive individuals such as for vaccine delivery?

Trono: For immunization, the presumption that the transgene will be delivered into immunologically normal cells. We do not know if a transient expression, or if long-term expression, would be better for producing an effective immunization.

J. Wilson: Is there any conceivable way in which an HIV vaccine could somehow subvert the therapy? I do not see if we have a pseudotype vector, it would be a shame to eliminate this possible vector for treatment or immunization against HIV.

Trono: But there is so little of the HIV remaining in the vectors.

Kingsman: Some protein carried in can get produced results in cytotoxic T-cell response.

Naldini: How much of what has been done on non-HIV vectors is based upon HIV? Do we actually know ...there are differences in different tissues; clearly the host range is playing an important role. We do have an excellent GAG and POL assay, and excellent drugs which could be used.

Kingsman: If we want to get them into the clinic, the HIV experience is compelling.

Audience: Have other pseudo typed vectors been tested?

Reiser: The alternative envelopes have been effective, but generally results are 1-log lower fold.

Trono: NEF helps in removing the envelope for the HIV receptor, and if the virus enters by vector-cell member fusion. If entry is derived by fusion with endosomes, the lack of NEF makes it more infectious.

Audience: Do any of the experiments of Garry Nolan? Do the FIV experiments use cyclosporin A?

Nolan: Cyclosporin was not used for two reasons, a primary one being the confounding observations to date.

Session II. Manufacturing, Bio-Safety and Testing
Roundtable Discussion

Chair:
R. Scott McIvor, Ph.D.

Panelists:
C. Estuardo Aguilar-Cordova, Ph.D.
Dale G. Ando, M.D.
R. Paul Johnson, M.D.
Douglas J. Jolly, Ph.D.
Luigi Naldini, M.D., Ph.D.
Anne M. Pilaro, Ph.D.
Carolyn Wilson, Ph.D.
Michael S. Wyand, D.V.M., Ph.D.

Audience: Is the VSVG a standard VSVG; and how likely is that it could occur being cytolytic?

Naldini: It is the Indiana 'cytolytic' type.

Ando: The probability would seem low; however, the potential for bringing in a new gene to patients and change in tropism of any recombinant would be important. That is why we would want to test.

Verma: A key question would be to answer the probability of generating a recombinant virus. *In vitro* this might be significant; *in vivo* it would seem unlikely.

Jolly: *In vivo*.

McIvor: Perhaps this is getting ahead of the discussion. What would be the relevant gene to test for, and what would be the relevant test?

Jolly: Testing for VSVG would be appropriate.

Naldini: GAG-POL is going to be present in a replication competent recombinant, and for which should be tested. However, we do not have a relevant test. When we pseudotype with VSVG, we are increasing the changing the infectivity by 1,000-fold.

Trono: What would be the infectivity of SIV pseudo typed with VSVG. An experiment is 'in progress' and completed *in vitro*. The replication appears to be decreasing. This virus has been inoculated into monkeys and results will be forthcoming. To the FDA officers, what would be the way for which to test?

C. Wilson: The requirements would not be more stringent than are used for Moloney MLV vectors, acknowledging that endogenous retrovirus sequences are present in the human genome.

Trono: So one would be testing for the transgenes, and not HIV endogenous

sequences.

Audience: In our experience with high concentration the lentiviral vectors we are studying, recombinant virus has been detected.

Trono: The presence of the tat protein may allow for an infection, but it is not a recombinant. We need to know the details of the experiment to interpret the results.

Audience: How do you work with them as wild type HIV, or as MLV?

Naldini: Originally, lentiviral was handled as a BL3 vector, but with more experience and inclusion of safety features, we are working with more relaxed conditions. Now we are facing the embarrassing situation of not having good tests for recombinants.

Jolly: Because we have a lot of experience with HIV, we have been doing this work-up until now in BL3. The most concerning situation is now with plasmids where we have had contamination of MLV preparations with infectious contaminants.

Trono: The nature of the transgene may be important, for example, an oncogenic transgene may be important and should be considered in establishing the biosafety level.

Audience: Regarding the SIN vector, inclusion of safety factors is often associated with decreased efficacy.

Naldini: In the *in vivo* studies, the SIN vector has remarkably more efficient expression (e.g., in the brain). In any case, safety should be the most important consideration factor in the early stages of work.

W. French Anderson: Why are you jumping on a San Francisco trolley car which is more dangerous?

Jolly: This was, in at least major part, a systemic approach. It was easier to maintain a uniform policy. We are itching to decrease the biosafety containment procedures.

Audience: However, this may be a dangerous time when so many different manipulations being done particularly with the VSVG recombinant (as in the case of the SIV-VSVG pseudotyped replication-competent recombinant).

Verma: We have done over 120 preparations and tested them 2 or 3 times with the various tests that were mentioned earlier, we have never seen recombination. That’s what we are telling people to do now.

Wilson: What kind of models would be useful at this point? As a community, there should be more discussion enabling some uniformity.

Johnson: It depends upon what type of work is being done. In going for proof of concept, or proof of product? Based on this one might choose the Macaque model, or not. But this many not be the best model for RCR.

Wyand: There are many different models that can be used in the preclinical phase, but may not be used for development of every product.

Audience: Perhaps use of the lentiviral envelope, and gain more information about the consequences of recombinants.

Trono: In fact, such experiments have been conducted, and the results are not different than one would expect. The titers are comparable (to MLV) until one tries to concentrate the vector for which it is unstable.

McIvor: Is there a consensus that, based upon the number of modifications being made, and in comparison to the vaccine work being done, that this is not more risky than the vaccine.

Wyand: It does appear that we will be able to create an attenuated SIV that is replication-competent and yet safe. In comparison, the lentiviral vectors are a significant degree away (safer).

Aguilar-Cordova: A partial recombination during production such that the vector contains VSVG, with superinfection, is there a possibility that a VSVG-pseudotyped HIV could then be generated in vivo?

Verma: This seems extremely unlikely based upon.

Naldini: This is the kind of thing that would need to be assayed in producer cells lines during the manufacturing process.

Trono: Consider what is occurring in nature, for example people co-infected with HTLV and HIV. We have never seen recombinants. There are such ‘dooms day’ ideas which are happening all the time in our environment, but recombinants have not occurred.

Verma: But have we ever looked? But lets put that into perspective. We have done 15 years of work to assess the MLV systems. The questions may not be different, and many of these questions regarding lentivirus are anticipated by our work with MLV.

Audience: What is the intellectual property status on these vectors, with so much?

McIvor: The safety and efficacy issues are the specific charge of this meeting and, with such little time left (Verma: and lack of expertise on patent issues), perhaps we should defer that question infavor of the pressing assignment.

Johnson: What about potential mobilization of vector in non-human primate model

systems?

Ando: What one finds *in vitro* may not be what one finds *in vivo*. That is the issue that should be broached now, what *in vivo* assays should be developed?

Johnson: How will the FDA respond to that?

Pilaro: Let's see the data. While safety is the major concern, we do not want to slam the door inappropriately. The specific results, the genes and disease under scrutiny, and the specific test results will have to be considered.

McIvor: Regarding such testing patients receiving lentiviral vectors, will the patient sero-convert thus eliminating the utility of such testing?

Jolly: We have actually run into that situation. Vector sensitization should be distinguishable by the results of Western and ELISA tests.

McIvor: In view of the fact that the RAC will be developing a consensus report on lentiviral vectors, are there any recommendations from this meeting to be taken to the RAC for consideration?

Verma: Only that you be assigned to head this project.

McIvor: If there are no other comments, let me take this opportunity, to thank the participants and bring this meeting to a close.