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Overview

Components of Gene Therapy Experimentation That Contribute to Relative Risk

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Gene therapy is the purposeful delivery of genetic material to somatic cells for the purpose of treating disease or biomedical investigation. Either viral or non-viral vector methods can be used. The risk of collateral exposure of laboratory animal care personnel to gene therapy vectors is dependent on a number of factors. These factors are intrinsic to the gene therapy vector (the vehicle for genetic conveyance), product encoded by the genetic construct delivered, method of delivery, and immune status of the recipient. The component risks of gene therapy experiments can be analyzed to surmise the overall relative risk of the experiment. Knowledge of the components that contribute potential hazardous risk to a study can assist animal care staff in identifying area(s) where prudent practices should be focused. Gene therapy experiments involving viral vectors are generally performed at either biosafety level 2 or 3. The objective of this review is to report on various components of gene therapy experiments, focusing on characteristics of viral and non-viral vectors, to assist the laboratory animal science community in determining prudent biosafety practices.

Introduction

In 1984, W. French Anderson proposed that a method of transfer of genetic material might be used in clinical therapy of human diseases (1). The purposeful delivery of genetic material to humans or animals as a method of medical intervention and biomedical investigation has come to be known as gene therapy. Gene therapy comprises various methods of delivery of exogenous ribonucleic or deoxyribonucleic acid (RNA or DNA), in specific or nonspecific manner, to somatic cells, resulting in alteration of cell phenotype. The process of delivery of exogenous genetic material into mammalian cells is called transfection. The vehicle used for the delivery of the therapeutic gene (hereafter referred to as a transgene) is called the gene vector, implying unidirectional transfer of material. The nature of the vector can be categorized as viral or non-viral. If the vector is a virus, it is either replication competent (infective) or deficient. Although there have been more than 250 clinical trials of gene therapy in humans, it wasn't until recently that the first "proof of principal" was documented by cure of a genetic disease (2). Retroviral gene transfer successfully delivered a functional copy of the human common gamma chain gene into $CD34^+$ cells that were transplanted into human infants afflicted with X-linked severe combined immunodeficiency (SCID-X1). Subsequent to this therapy, two of 10 children developed leukemia that was directly attributed to the gene transfer (3, 4). In an unrelated gene therapy clinical trial, the death of a recipient of high-titer adenovirus was directly attributed to the gene therapy (5).

Growth in the number and complexity of gene therapy experiments in laboratory animals has been fundamental to progress in the field. However, safety precautions are warranted for laboratory animal personnel working in the vicinity of gene vectors or laboratory animals that are the recipients of gene therapy. The purpose of the article reported here is to review gene therapy fundamentals to understand how various aspects contribute to the overall risk for animal care personnel. Risk analysis for a gene therapy study is inherently subjective and is similar to risk analysis for experiments involving any biohazardous agents: dose of the agent (concentration and volume), route of exposure, infectivity of the agent, mode of transmission, and immune status of the host. For most gene therapy experiments, consideration of the biological properties of the gene vector are substituted for virulence of a microbial agent. Biological properties of the vector include stability of gene after transfer, control of gene expression, and function of the therapeutic protein (or RNA) it encodes. Table 1 outlines the components of a gene therapy experiment that need to be considered.

The US Government through the National Institutes of Health published *Guidelines for Research Involving Recombinant DNA Molecules* (59 FR 34496 June 24, 1994, as amended, referred to as the *Guidelines*). The *Guidelines* provide a framework, within which molecular biology research and related disciplines, such as gene therapy, would be performed in the United States. These activities are overseen by the Office of Biotechnology Activities at the National Institutes of Health. The *Guidelines* define the role and composition of an Institutional Biosafety Committee (IBC) to be formed at relevant research institutions to provide oversight for certain categories of recombinant DNA experiments. Each principal investigator must initially assign a biosafety level to the gene therapy experiment, which the IBC accepts or modifies at a convened meeting. The use of risk group-2 agents that contain greater than two-thirds of their genome must be performed at biosafety level (BSL-) 2. However, what is lacking in the *Guidelines* is emphasis that the

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Table 1. Components of a gene therapy experiment that need to be considered in determining the relative risk of inadvertent personnel exposure to vectors

I. The vector
  A. Viral
    1. Replication-competent vs. replication-deficient
      a. Potential reversion to replication-competence
      b. Viral shedding—active and/or passive
    2. Cell tropism
    3. Transient vs. stable expression transfection
      a. Stable—epichromosomal, random integration, site-specific integration
    4. Vector titer (concentration)
    5. Expression of other viral vector proteins
    6. Potential for recombination with wild-type viruses
  B. Non-viral
    1. Receptor-mediated endocytosis
    2. Liposomes
    3. Naked DNA
    4. Microprojectile
    5. Other
II. The expression cassette
  A. Transgene–structural protein, enzyme, toxin, cytokine, peptide hormone
  B. Promoter–constitutive vs. inducible, tissue specific vs. generalized
  C. Level of gene expression
III. Method of delivery
  A. In vivo
  B. Ex vivo
  C. Physical properties–aerosolized, microprojectile (ballistic), parenteral injection, or catheter-based delivery
IV. Health status of the therapy recipient
  A. Healthy vs. diseased
  B. Immune competent vs. immune deficient

assignment of biosafety level to a gene transfer experiment must take in account all aspects that contribute risk of gene transfer to laboratory personnel.

Risk analysis in gene transfer experiments

Gene transfer experiments can be dissected into various component risks for evaluation of overall experimental risk to research personnel: gene to be transferred, vehicle for gene delivery (vector), physical method of delivery, and immune status of the recipient. The expression cassette, composed of other nucleic acid sequences accompanying the gene, determines the magnitude, temporal, and spatial aspects of gene expression. Vector properties determine which cell populations will be gene recipients, stability of the genotype, and the potential for horizontal gene transmission. The method of vector delivery determines the potential for personnel exposure due to aerosol generation or other physical risks. One simplistic method to determine relative risk is to assign a numerical score to each component risk, thereby deriving an arithmetic total of overall experimental risk as shown in Table 2.

Component risks in gene transfer experiments commonly have not been evaluated; thus, experiments using viral vectors are performed at BSL-2. Experiments using non-viral vectors are performed at BSL-1. However, when consideration is given to all experimental components that contribute risk, we might consider placing these experiments on a scale: for viral vectors, a spectrum from BSL-2 to BSL-3; and for non-viral vectors, from BSL-1 to BSL-2. The decision to assign prudent biosafety practices requires knowledge of each experimental aspect that contribute to overall risk. This review serves to acquaint the reader with information regarding the various component risks in gene transfer experiments.

Gene therapy in research and molecular medicine

Gene replacement to correct inborn errors of metabolism was the first nucleic acid-based therapeutic strategy conceived (1, 6). Successful therapy would lead to production of the desired protein at a physiologically relevant level of expression, if necessary, limited to a particular cell type. As the field of gene therapy matured, other potential uses were conceived in many fields of medicine. For example, to prevent cell proliferation in neoplasia or inappropriate cellular hyperplasia, several groups proposed delivery of negative selectable markers genes (suicide genes) to be expressed in the offending cell type (7). Expression of suicide genes (e.g., cytosine deaminase or thymidine kinase [TK}) renders cells susceptible to the lethal effects of specific pharmacologic agents while non-transfected cells are spared. Expression of cytosine deaminase (8) or TK (9) induces susceptibility to the lethal effects of treatment with 5'-fluorocytosine or acyclovir, respectively. Expression of tumor suppressor genes, such as p53 (10), p16 (11), or the retinoblastoma proteins (12), has resulted in suppression of tumor growth and/or lead to tumor regression in laboratory animal studies. Thus, inadvertent exposure of personnel to suicide gene vectors would result in susceptibility of transfected cells to the lethal effects of these compounds, an overall risk that is minimal.

Alternative strategies to inhibit cell proliferation, such as overexpression of dominant negative receptors for growth factors to interfere with cell signaling, or use of transcription factor decoys to overwhelm transcription factor E2F-preventing gene transcription have been developed, resulting in cell death (13). Strategies designed to prevent the expression of genes have been developed, and include antisense therapy, delivering a

Table 2. Components contributing to relative risk of gene therapy experiments

High risk*					Low risk
Risk score	5	4	3	2	1
Gene	Toxin	Cytokine	Enzyme	Antisense	Structural
Vector cell tropism		Nonspecific		Tissue specific	
Duration of expression		Stable		Transient	
Level of expression		High			Low
Promoter of gene expression		Constitutive, non-cell specific	Constitutive cell specific	Inducible non-cell specific	Inducible cell specific
Aerosol generated during delivery		High			Low
In vs. ex vivo		In vivo			Ex vivo
Vector replication		Competent		Deficient	
Host immune status		Immune deficient			Immune competent
Score = 37					Score = 12

*Viral vector experiments are on a scale from biosafety level 2 to 3; non-viral vectors are on a scale from 1 to 2.

gene encoding a RNA complementary to a particular messenger RNA (mRNA) to prevent translation and protein expression (14). Similarly, ribozyme therapy (15, 16) delivers genes encoding RNA sequences with enzymatic activity to degrade a specific mRNA sequence, preventing translation and expression of a particular protein.

Route of exposure: in vivo and ex vivo gene therapy

Gene vectors can be administered to patients or laboratory animals by a number of routes: parenteral, topical, inhalation, surgical or catheter-based, intracerebroventricular, intra-parenchymal, intra-luminal, and ballistic. Each method of in vivo delivery exposes a different subset of cell types and populations to the gene vector, leading to differing topographies of transgene expression. For example, injection into the bile duct, compared with injection into the portal vein, leads to a different subpopulation of liver cells (bile duct epithelia, hepatocytes, resident macrophages [Kupfer cells], endothelial cells, and fibroblasts) expressing the therapeutic gene. Alternatively, tissues can be harvested from laboratory animals and maintained in culture for ex vivo gene therapy. Similar to the treatment of human SCID X-1 patients, cultured cells are exposed for a period to a gene vector in vitro, the vector is subsequently washed from the cell culture, and the genetically modified cells are re-implanted into the animal (2). The potential for animal care personnel to encounter a substantial dose of gene vector in the secretions or excretions of a recent ex vivo gene therapy subject is low, relative to the hazard represented by body fluids of the same subject after in vivo gene therapy. Methods of delivery that produce aerosolized vector or involve more extensive preparation of the vector pose greater risk.

Viral and non-viral vector systems

Vectors for gene delivery can be viral or non-viral. Viral vectors have limitations in the size of the gene they can convey that is determined by the viral capsid's internal volume (17). Viral vectors are either replication-competent or replication-deficient. The genetic material delivered by non-viral vectors does not have size limitation. Compared with viral vectors, non-viral vector systems are less efficient in transfecting cells. The efficiency of non-viral gene transfection diminishes rapidly as the size of the genetic material increases beyond 20,000 nucleotide base pairs (20 kb) (18). To put transgene size in context, the average size of mRNA is 7 kb, with some as large as 10 kb encoding a single protein. However, genes in chromosomes vary greatly in size, with many being > 20 kb. The risk of untoward exposure to non-viral vehicles is only relevant at the time of transgene delivery. The risk of exposure to viral gene vectors is during delivery and afterward; the time frame is dependent on the passive secretion of vector for replication-deficient vectors, and the period and route of shedding for replication-competent viral vectors.

Viral vectors. Viruses have inherent cell targeting and specificity (tropism), gene expression capabilities, and high transfection efficiency rates (19). Adenovirus, adeno-associated virus (AAV), retrovirus, lentivirus, Semliki Forest virus (SFV), and Sindbis virus are examples of replication-deficient gene vectors. Replication deficiency is induced by deletion or separation of portions of the viral genome, allowing production of empty viral capsids in a manner that precludes self-replication (19). The greater the size of deletion of the viral genome, the less the potential for the vector DNA to recombine in any manner that produces a replication-competent form. First-generation adenoviral vectors have small deletions in the gene encoding E1a protein, a gene necessary for viral replication, as well as a deletion in the noncritical E3 gene. First-generation adenoviral vectors are propagated in human embryonic kidney (HEK-293) cells. The HEK-293 cells are a continuous transformed cell line immortalized by incorporation of the adenovirus type-5 E1 gene (20). Prototype replication-deficient AAV vectors require co-infection with a helper virus (usually an adenovirus or herpesvirus) to obtain AAV replication (21). After AAV gene vector production, the AAV and adenovirus are heated to inactivate adenovirus, leaving the AAV vector capable of transfecting cells.

First-generation replication-deficient retroviral vectors are produced by helper virus rescue, requiring an additional step to separate vector from helper virus (21). However, newer viral vector systems have packaging cell lines that produce empty viral capsids encoded by genes physically separated between bacterial plasmids for adenovirus (22), retrovirus (23), lentivirus (24), SFV (25), Sindbis virus (26), and AAV (27).

Some viral vectors are inherently incapable of completing their replication cycle in nonpermissive cells. Baculovirus is an insect virus that can transfect mammalian cells, but is unable to replicate (19). Inoculation of laboratory animals with replication-deficient viral vector can only lead to horizontal transmission if there is passive loss of unbound vector (shedding) into body fluids, since replication does not occur. In cases where the potential for horizontal transmission was examined, substantial loss of high-titer vector was limited to the first day after inoculation and was undetectable by 72 h (28-30).

Replication-competent viruses that are used as gene therapy vectors include herpes simplex virus type 1 (HSV-1) and vaccinia virus (an orthopoxvirus) (31, 32). These viruses have a very large genome, compared with that of replication-deficient viral vectors, and can accept larger transgenes. Both HSV-1 and vaccinia viruses contain TK, an enzyme not found in most mammalian cells. The method of producing these HSV-1 and vaccinia vectors involves insertional inactivation of the TK gene by the transgene; TK is not critical to viral replication. However, subsequent treatment with acyclovir (thymidine analog) or 5'-bromodexoyuridine (5'-BrdU) only permits propagation of recombinant virions defective in TK expression (32). Introduction of a transgene into vaccinia virus and selection for recombinants is shown diagrammatically in Fig. 1. Wild-type HSV-1 or vaccinia virus that express TK incorporate acyclovir into their DNA, which prevents viral replication. When inoculated into animals these vectors replicate in permissive cells in manner typical of viral infection (32). There is amplication of the recombinant virus and the potential for horizontal transmission. Therefore, replication-competent viral vectors represent a relatively greater hazard, compared with that of replication-deficient vectors, due to the initial increase in viral titer from infection and prolonged period of viral shedding.

Viral vectors: stable versus transient gene expression. Retroviruses, lentiviruses, HSV-1, and AAV are capable of inserting a DNA copy of their genome into the genome of transfected cells, leading to stable expression of transfected genes potentially for the lifetime of the altered cell (19). Table 3 contains a summary of the tropism of these viral vectors and stabil-

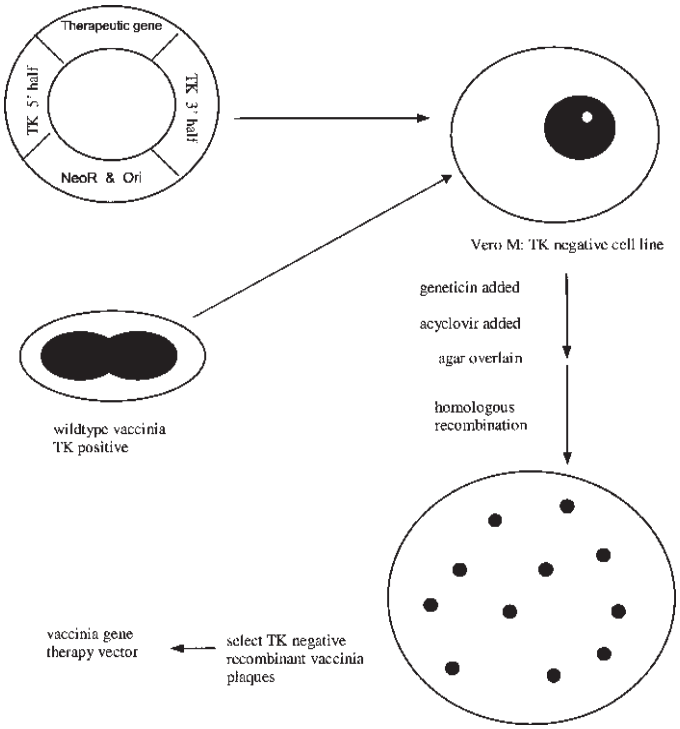

Figure 1. Diagram representing introduction of a transgene into a vaccinia viral vector. A similar strategy is used for herpes simplex type 1 (HSV-1) vectors. The Vero M cell line is transfected by use of a plasmid containing the transgene flanked by segments of the viral thymidine kinase (*TK*) gene. The plasmid neomycin resistance gene (*NeoR*) allows transfected cells to survive treatment with geneticin (G418). The transfected cell line is inoculated with wild-type vaccinia virus, and acyclovir is added to the culture medium. Cells in which vaccinia DNA underwent homologous recombination with plasmid DNA resulted in having the recombinant virus as the transgene caused insertional inactivation of *TK*. By overlaying the monolayer with agar, recombinant virus spread is limited and plaques (holes) grow in the cell culture monolayer. Wild-type vaccinia virus expresses *TK* and cannot replicate in the presence of acyclovir. Ori = mammalian and bacterial origins of replication.

ity of transgene expression. Retroviral vectors efficiently transfect only actively dividing (progenitor) cells, integrating a copy of their genome into the cells so that progeny cells also express the gene product (33). Retroviral and lentiviral vectors integrate into the host cell's genome in random manner. Wild-type AAV can infect either progenitor or terminally differentiated cells and integrate as a single copy into a specific site on human chromosome 19 (34). The HSV-1 is tropic for nerve cells and some epithelial cell types (31). In the adult, most neurons do not divide, thus having extremely long lifespan. Stable transfection of neurons by an HSV-1 vector could result in life-long transgene expression in neurons. However, during the latent phase, expression of genes in herpesviruses is strongly down regulated (31).

Adenovirus and vaccinia virus vectors bind to terminally differentiated cells, and the transfected DNA assumes the form of an epi-chromosomal body that is degraded by intracellular enzymes (35). The duration that transgene expression can occur is determined by two processes, cell death or transgene degradation. When adenoviral vectors have been used for gene transfer into neurons, degradation of the transgene occurred in 45 to 60 days (36, 37). The HSV-1, vaccinia virus, and first-generation adenoviral vectors express large amounts of endogenous viral

Table 3. Characteristics of some viral gene therapy vectors

Virus type	Genomic size (kb)	Tissue tropism	Transient (T) or stable (S)
Adenovirus	36	Lung, intestine, kidney	T
AAV	4.7	Many types	S
Herpesvirus	152	Epithelial–brain, skin	T
Semliki Forest virus	11.4	Many types–mammalian, reptilian, insect	T
Sindbis virus	11.7	Dendritic cells	T
VEE virus	11.4	Dendritic cells	T
Vaccinia virus	186	Epithelia–liver, lymphocytes, lung, skin	T
Retrovirus	9.2	Bone marrow, lymphocytes	S

AAV = adeno-associated virus; VEE = Venezuelan equine encephalomyelitis virus

proteins in addition to the transgene since much of the viral genome is delivered with the transgene. Viral proteins that are expressed on the surface of transfected cells lead to immunologic attack. Immune recognition of viral vector transfected cells is advantagous if the purpose of gene therapy is destruction of neoplastic cells or vaccination when viral proteins may act as adjuvant (38). Experiments involving viral vectors capable of stable gene transfer are potentially more hazardous than are those using vectors that induce transient gene expression.

Retroviral vectors. Murine amphotropic retroviruses have been developed as gene therapy vectors with broad species tropism (35). A variety of packaging cell lines that produce empty retroviral particles are commercially available. Each packaging cell line contains either one or two copies of the retroviral genome, in which the ψ encapsidation signal has been removed and the long terminal repeats (LTR) have been altered or deleted to prevent recombination events that may lead to production of wild-type retrovirus (39). The greatest limitation of retroviruses as gene therapy vectors is their small capsid size, which limits the size of the therapeutic gene insert to < 8 kb. Compared with other viral vectors, retroviral vector systems produce relatively low levels of stable gene expression. Retroviral tropism for progenitor cells can result in lifelong expression of a transgene after a single transfection event.

A disadvantage of retroviral vectors for gene therapy is random integration into the host genome at multiple sites, potentially inducing gene mutations as they do so, either by knocking out endogenous genes or altering their expression through promoter activity of the viral LTR. Integration of a retroviral vector near the *LMO2* locus induced leukemic transformation of a subset of $CD34^+$ cells in two of 10 patients receiving gene therapy for SCID-X1 (4).

Retroviral vector packaging cell lines that have been engineered to express coat proteins of other viruses result in vectors with broader cell tropism. Table 4 lists the alteration of species tropism due to expression of vesicular stomatitis virus (VSV-G) surface glycoprotein in a commercial retroviral gene vector kit (Retro-X, BD Clontech, Palo Alto, Calif.). Since retroviral gene constructs have less than two-thirds of the viral genome and are not efficiently aerosol transmitted, they could be classified as BSL-1 handling. However, manufacturer's recommend handling retroviral vectors at BSL-2 because of their random integration into the human genome. Retroviral vector kits are commercially available (Retro X, BD Clontech, and PvPack, Stratagene, La Jolla, Calif.).

Lentiviral vectors. Vectors derived from lentiviruses, such as human immunodeficiency virus type 1 (HIV-1), are capable of

Table 4. Retroviral vectors available from BD Clontech (Palo Alto, Calif.)

Target cell	pVPack-Eco	pVPack-Ampho	pVPack-10A1	pVPack-VSV-G
Mouse	+	+	+	+
Rat	+	+	+	+
Hamster	-	±	+	+
Rabbit	-	+	ND	+
Mink	-	+	+	+
Cow	-	±	ND	+
Cat	-	+	+	+
Dog	-	+	+	+
Monkey	-	+	+	+
Human	-	+	+	+
Chicken	-	±	ND	+

Variation of species tropism by surface glycoproteins from company literature. + indicates good transfection efficiency, ± indicates reduced transfection efficiency, and - indicates poor efficiency.
ND = not determined.

transfecting dividing and non-dividing cells (27). The ability to infect non-dividing cells is a major advantage over that of retroviral vectors, which are tropic only for dividing cells. The HIV-1 is tropic for $CD4^+$-expressing cells, such as macrophages, dendritic cells, some T lymphocytes, and some dividing epithelial cells of the gastrointestinal tract. Replication-defective vectors based on HIV-1 core proteins are extremely stable, and when produced by co-expression with VSV-G, manifest expanded cell tropism for neurons, glia, airway epithelia, hematopoietic stem cells, fibroblasts, and muscle cells (33). Replication-deficient HIV-1 vectors are deficient in the genes *tat* and *rev*, and the accessory genes *vif*, *vpr*, *vpu*, and *nef*. These genes are required for HIV-1 replication and, additionally, act as virulence factors (33). Increased stability of the packaging cell lines was achieved by eliminating these genes since their products are lethal to cells (33). Appreciable improvement in the biosafety of HIV-1 vectors has also been achieved by engineering self inactivation (40). These self-inactivation vectors lack the U3 region of the HIV-1 LTR. During normal replication of retroviruses, the upstream U3 region is replaced by downstream U3 during the reverse transcription process. The U3 region is the normal promoter of HIV-1 gene expression. Transgene expression at moderate levels has been achieved by removal of the U3 promoter and incorporation of a synthetic promoter.

Adenoviral vectors. Gene therapy vectors have been developed and are based on human adenovirus types 2 and 5, which are associated with human upper respiratory tract infections. These adenoviruses belong to subgroup C, which unlike other subtypes of human adenoviruses, lacks oncogenic activity in assays on BHK-21 cells (41). The adenovirus genome is larger (≤ 36 kb) than that of retroviruses; therefore, the capsid can accept a larger transgene. Adenoviruses are tropic for terminally differentiated epithelial cells transiently expressing relatively high levels of transgene products, compared with those of retroviral vectors.

The most commonly used adenoviral vectors have El and E3 gene deletions. These adenoviral vectors propagate in El transformed HEK-293 cells available from the American Type Culture Collection (ATCC, Manassas, Va.). The E3 region encodes a protein that helps the virus evade the immune system. Gene function of E3 is not essential for adenoviral replication (35). First-generation adenoviral vectors are capable of accepting a transgene up to 8 kb in size in either the El or E3 regions.

Replication of El-deleted adenoviral vector can occur after transfection of bone marrow (42). Bone marrow, and perhaps other tissues, express trans-acting factors that complement the E1-deletion deficiency of first-generation adenoviral vectors. Infection of epithelial cells with E1-deleted adenoviruses at high multiplicity of infection can overcome the block to adenoviral replication (43). Replication of E1/E3-deleted adenoviral vectors occurs during co-infection with wild-type adenoviruses of subgroups A, B, or C that supply the E1 factor necessary for recombinant vector replication (44, 45). Newer adenoviral vectors have genomic deletions in the E1, E2, E3, and E4 regions, and are less likely to replicate despite trans-acting cell factors or co-infection with wild-type adenoviruses.

Subsequent to adenoviral vector administration in immune competent mammals, there is pronounced attack on the transfected cells due to the expression of endogenous adenoviral proteins (e.g., hexon, penton, and fiber) in addition to the therapeutic gene product. Adenoviral vector delivery in utero for gene transfer into the tracheal epithelium of fetal lambs led to reactive hyperplasia and squamous metaplasia, with intense inflammation of the tracheobronchial tree and distal pulmonary alveolar spaces (46). An anamnestic immune response to adenoviral proteins, resulting in an immediate intense reaction, contributed to disseminated intravascular coagulation and multiple organ failure in one person in an adenoviral gene therapy trial (5).

Limited studies on passive adenoviral vector shedding have been performed. Passive excretion of the viral vector is dependent on the method of delivery, organ targeted, and species inoculated. In general, passive adenoviral vector shedding in the urine and feces occurs for < 72 h after inoculation (29). In mice, intravenous administration of the vector results in preferential delivery of the therapeutic gene to the liver without detectable shedding of vector. Transrectal administration to the prostate gland in a canine model led to detectable shedding of vector in the feces for 48 h (47). Aerosol delivery to human lung resulted in transient expression and no detectable shedding of adenoviral vector in sputum (48).

Routine cagewash temperatures achieved during the washing phase (74°C) and final rinse (82°C) are sufficient to inactivate adenoviral vectors (49). Adenoviral vectors are commercially available as kits (AdenoX, BD Clontech; AdEasy, Stratagene; and Adenovirus Expression Kit, Panvera Corporation, Madison, Wis.). Most adenoviral vectors that contain greater than two-thirds of the viral genome are handled at BSL-2, including those commercially available.

Adeno-associated virus. The AAV is a replication-deficient parvovirus, of which six serotypes with varying cell tropisms have been identified (41). Serotype AAV-2 is most commonly used as a gene vector and has not been associated with naturally acquired disease in humans (50). The AAV-2 genome is small (< 4.7 kb), and the capsid volume limits the size of a gene insert to < 4 kb. The AAV is capable of transfecting actively dividing and quiescent cells (50). The AAV-2 genome integrates into a specific site on human genome chromosome 19 (AAVS1) (34). To the author's knowledge, there are no reports that document stable transfection in animal cells. It appears that a homologous site to AAVS1 does not exist in animals. The AAV vectors will integrate into AAVS1 transgenic mouse and rat models (34). First-generation AAV vector production requires helper virus in the form of an adenovirus or a herpesvirus, as discussed previously (41). A helper virus-free packaging system has been developed and is commercially available (51).

Intramuscular inoculation of AAV into rhesus monkeys re-

sulted in detectable infective particles in the blood for 48 to 72 h after inoculation. The AAV vectors contain less than two thirds of the viral genome, warranting BSL-1 practices (28). Further precautions may be indicated depending on the charateristics of the transgene.

Sindbis virus and SFV. These replication-deficient vectors are based on positive-sense single-stranded RNA alphaviruses (52). A transgene ≤ 7 kb in size can be placed into either system with efficient packaging. The extremely high level of expression of the therapeutic gene occurs 48 h after inoculation, leading to death of the transfected cell (53). At that time, up to 25% (SFV) or 80% (Sindbis virus) of the total cellular proteins are from transgene expression (54). Non-cytopathogenic vectors have been constructed for SFV and Sindbis virus on the basis of point mutations in the non-structural protein 2 (nsP2) (53, 55). These vectors induce appreciable gene expression, but result in substantially longer survival of host cells, compared with conventional SFV vectors (53, 55). Other point mutations or deletions in the nsP2 gene have lead to novel replication-competent vectors that persistently infect host cells. The SFV is tropic for neurons. Intranasal or intravenous admiministration of SFV results in transgene expression in the central nervous system (25). Immunologic response to the SFV vector is minimal after repeated administration (53).

Early SFV replication-deficient vectors were associated with small amounts of contaminating replication-competent virus. To prevent replication competence, an SFV mutant in the surface glycoprotein (E2 spike protein) was produced. This mutant was resistant to proteolytic cleavage of the E2 protein, which is a critical step in development of infectivity (54). The peptidase-resistant mutant vector remained non-infective until it was treated with chymotrypsin (56). The SFV vector is commercially available (Invitrogen, Carlsbad, Calif.); however, a report of a fatal laboratory exposure to wild-type SFV led the manufacturer to recommend handling SFV vector at BSL-2.

Herpesvirus vectors. Herpesviruses are so large (≤ 150 kb) that they cannot be engineered in the manner of whole genome manipulations described for retroviruses and adenoviruses (17, 31). Infection with HSV-1 is associated with fever blisters or canker sores in humans. Most experiments with HSV-1 vectors involve use of those that are replication competent. Genes transferred to peripheral nerve endings, using HSV-1 based vectors, are transported centrally and expressed (57, 58). The immune response to HSV-1 vector-transfected cells is similar to that of the adenoviral vector system; however, there is more inherent toxicity of endogenous viral-encoded gene products associated with the HSV-1 system (59).

A replication-deficient HSV-1 mutant has been developed by deletion of the *IE3* gene (60). This vector can be propagated in an *IE3* gene-modified cell line. A neuro-attenuated ICP34.5 HSV-1 variant also has been developed that replicates in ependymal cells after intracranial inoculation in mice without causing encephalitis (61). A replication-deficient HSV-1 vector developed by removal of the genes encoding the infected cell protein (ICP) 22 and ICP27 has been described (62). A cytomegalovirus (gamma herpesvirus)-based viral vector recently has been developed (62). Human cytomegalovirus is tropic for hematopoietic progenitor cells, lymphocytes, and salivary gland epithelium.

Intracerebroventricular inoculation of replication-deficient HSV-1 in owl monkeys did not result in detection of the virus in tears, saliva, or vaginal secretions by use of polymerase chain reaction analysis at any time up to one month after inoculation (30). In that study, anti-HSV-1 antibodies developed over a period of 21 days after inoculation. The HSV-1 vectors contain greater than two thirds of the viral genome and are handled minimally at BSL-2.

Vaccinia virus vectors. Vaccinia virus is an orthopox virus commonly used to transfer genes into a variety of mammalian species (32). During the 1960s and 1970s, people were inoculated with vaccinia virus in a world-wide smallpox eradication effort. The origin of vaccinia virus is unknown, but it is thought to be a variant of the cowpox virus first used by Jenner in his development of the smallpox vaccine. Vaccinia virus is not associated with naturally acquired disease in humans.

Vaccinia virus gene vectors are produced in manner similar to production of recombinant HSV-1 vectors. The large genome (186 kb) of this virus allows insertion of substantially larger transgenes than does that of replication-deficient vectors. Compared with HSV-1, vaccinia virus has broad species and tissue tropism (lung, liver, spleen, and skin). It is characterized by high transfection efficiency and transient gene expression (32). Transgene expression after recombinant vaccinia virus inoculation occurs for approximately seven days. Recently, replication-deficient vaccinia virus vectors have been produced by mildly treating the competent vector with psoralen and ultraviolet light (63, 64).

Recombinant vaccinia virus has been used extensively in vaccine production. Vaccinia inoculation elicits a profound immunologic response due to viral replication and expression of endogenous vaccinia virus proteins that act as adjuvant. Subcutaneous inoculation of strain-13 guinea pigs with recombinant vaccinia at three- to four-week intervals for three administrations did not lead to seroconversion of non-inoculated cagemates (65). Intradermal inoculation of recombinant vaccinia virus in calves led to high-titer virus and lesion development. Furthermore, recombinant vaccinia virus isolated from lesions failed to induce lesions when passaged in a second group of calves inoculated by the same route (66). Inoculation of mice with vaccinia virus by subcutaneous injection and intrarectal instillation led to transmission to only one of 15 sentinel mice in direct contact with the inoculated animals (67). Transmission did not occur to sentinel mice receiving soiled bedding from cages of the inoculated cohort. Vaccinia virus vectors contain greater than two thirds of the viral genome; therefore, they are handled at BSL-2.

Nonviral vectors

Non-viral gene transfer to cells induces transient gene expression in specific or nonspecific manner. Specific cell targeting can be conferred by use of direct DNA injection, or linking the therapeutic gene to a ligand for specific binding to a cell surface receptor. The DNA delivered by nonviral means exists as an episomal element, unless the therapeutic gene is flanked by DNA sequences that can undergo homologous recombination within the host cell's genome. Homologous recombination is an extremely rare event, so few transfected cells will stably express the therapeutic gene. Non-viral vectors are handled at BSL-1, unless there is risk associated with the transgene and/or method of delivery that warrants more stringent biosafety practices. Hazards to personnel associated with non-viral gene

transfer are associated with the method of delivery, and include such possibilities as accidental hypodermic needle stick, direct inoculation by ballistic methods, and mild chemical burns that can be associated with some transfection formulations. To the author's knowledge, there have been no reports of horizontal transmission of transgenes administered by use of non-viral vectors. The following sections are provided to familiarize the reader with commonly used methods of non-viral gene therapy.

Receptor-mediated endocytosis (RME). Introduction of a transgene into a particular cell type demands a system that specifically targets the DNA construct for binding and uptake by the desired cell population. This method provides a means to accomplish this transgene cell-targeting process (68). Cells use RME to take up molecules from their environment as part of a variety of biological processes, including cell signaling (cytokines, transmitters, growth factors) and uptake of transmembrane carrier proteins (transferrin and low-density lipoprotein [LDL]). After a ligand binds to its cell surface receptor, the complex is internalized via clathrin-coated pits. The complex enters the endosomal compartment, which becomes increasingly acidic internally. The endosome fuses with a lysosome, and the contents are degraded. The fate of the endocytosed complex is dependent on the physicochemical nature of its contents.

Covalent linking of DNA to ligands, such as asialoglycomucoproteins or ferritin, becomes selectively bound to hepatocytes and is internalized with subsequent expression. Oher cellular receptor-specific ligands that have been linked to DNA gene constructs are insulin, lactosylated polylysine, antibodies (e.g., antithrombomodulin, anti-CD4), recombinant HIV GP120, and epidermal growth factor (68). The transgene is linked to the ligand by use of a polycation, such as poly-lysine or protamine, or via a bifunctional reagent, such as N-succinimidyl 3(2-pyridyldithio)-priopionate. Polycationic reagents are more efficient in cell transfection due to their compact complexing of DNA into toroidal structures maximally condensing. Then minimizing molecular volume. Polycationic linkers react ionically with DNA and do not require covalent linkage (17).

The hemagglutinin (HA) protein of influenza virus and the fusogenic protein of Sendai virus have been covalently linked to the ligand-DNA complex to prevent degradation in the phagolysozome (69). These viral proteins promote disruption of the endosome, releasing the transgene into the cell cytoplasm (69). The capsid of adenovirus linked to poly-lysine DNA complexes increases the efficiency of cell transfection. The adenovirus capsid mediates efficient binding to the cell surface and imparts a mechanism of endosomal escape (70). Synthetic peptides that mimic HA protein also facilitate endosome escape (69). The quinone antibiotic chloroquine precludes acid pH in the endosomal compartment. Chloroquin administered concurrently with RME gene therapy protects the transgene from degradation since lysosomal enzymes require an acid environment to function (18, 70).

Liposomes. Liposomes contain anionic or cationic lipids that are categorized by the ionic charge of the polar head region. Lipids have a middle bridge region and a hydrophobic anchor region. Lipids are further classified into three categories on the basis of the anchor region: single acyl chain, double acyl chain, or cholesterol (18). Cationic lipids are the most commonly used non-viral gene therapy vector. Cationic lipids interact with the negatively charged phosphodiester backbone of the DNA as well as the negatively charged proteoglycans on the surface of cell membranes, delivering transgenes without cell targeting specificity. Depending on the ratio of DNA to lipid, the liposomes may form micelle-like, toroidal, or rod-like structures. Cationic liposomes are usually combined with dioleolphosphatidylenthanolamine (DOPE), facilitating cell transfection (18). Liposomes fuse with the cell's plasma membrane, discharging the transgene into the endosomal compartment. The DNA must be discharged from endosomes, a process facilitated by the addition of DOPE to the liposome. Ligands can be covalently bound to anionic lipids, conferring tissue specificity to gene delivery (72). Once discharged into the cytosol from the endosome, the DNA is transported into the nucleus through the nuclear pore complex. The lipids must be removed from the DNA before gene expression, a process that occurs when the lipid complex escape from the endosomal membrane.

Anionic lipids are far less efficient in liposomal gene therapy; however, ligands more easily form ionic complexes with anionic lipids, resulting in specific cell targeting (71). Anionic liposomes have less non-specific ionic interaction with the negatively charged cell membrane than do their cationic counterparts (71).

Cationic liposomes are an efficient method for transgene delivery, and have been used in vivo and in vitro with DNA and RNA. Intravenous liposomal gene delivery leads to transfection of all major organs, including heart, lung, liver, spleen, and kidney. The lungs consistently have the highest levels of gene expression (71, 72). Transgene is expressed in most cells for fewer than seven days. Liposomal delivery of mRNA leads to expression for one to two days. Liposome-mediated transgene delivery in skeletal muscle has been reported to last up to 28 days. Lipopolyamine-complexed transgene given intravenously to third trimester mice in late pregnancy resulted in gene transfer to developing embryos (73). Transgene expression continued in the newborn progeny.

Direct transgene injection. Direct DNA injection of the luciferase reporter gene into skeletal muscle was the first documentation of transgene expression by use of naked DNA injection (74). Injection of DNA has been further developed as a method of vaccination for viral disease prophylaxis (7). Polymers, such as polyvinyl pyrrolidone, complexed with transgene improve the efficiency of gene transfer. Intravenous injection of plasmid DNA results in limited gene expression in liver, lung, kidney, spleen, and heart (75). Rapid high-volume intravenous injection of DNA in the tail of mice efficiently tranfected liver cells (76). This increased efficiency appears to be mediated by an active receptor uptake mechanism, which becomes even more efficient if the caudal vena cava is temporarily occluded immediately after injection (77).

Vaccination by use of direct DNA injection does not induce an immune response to the genetic material (DNA or RNA), only to its protein product. Repeat vaccinations are not hindered by immune memory of the proteins expressed by the vaccination (78). Cells that have an inherently lower turnover rate (muscle and neurons) express the therapeutic gene for one month or longer following direct DNA injection.

Microprojectile delivery of a therapeutic gene. Microscopic tungsten or gold particles (one to three micrometers in diameter) coated with precipitated therapeutic gene can be "shot" at high velocity into tissues, leading to gene expression. The Accell system (Powderject Pharmaceuticals, Inc., Madison,

Wis.) and Helios (Bio-Rad, Hercules, Calif.) are commercial systems available for performing this method of gene delivery (80). Mitochondria contain DNAs encoding intrinsic mitochondrial proteins. Maternal inheritance is the sole method of between-generation transmission of mitochondrial DNA, which is present in the oocyte at the time of fertilization. Microprojectile transgene is the only method developed thus far that will deliver a transgene to mitochondria (79). A variety of genetic diseases result from mutations in mitochondrial DNA that might be amenable to microprojectile gene therapy (80). From a practical standpoint, microprojectile transgene delivery is limited to the skin. Cutaneous microprojectile DNA vaccination takes advantage of the high concentration of antigen-presenting Langerhans cells in skin to elicit T lymphocyte- and humoral-mediated immune responses (81).

Therapeutic gene constructs

The expression cassette. The DNA sequences used for gene therapy contain a series of non-coding components other than the transgene to be expressed. Non-coding DNA sequences regulate the level and tissue specificity of gene expression. When constructing the expression cassette, amino acid sequences can be incorporated into the expressed protein that regulate the intracellular localization of the protein or its secretion into the extracellular environment. It is necessary to understand the control, release, and function of the transgene product to determine the relative risk associated with gene therapy.

The full complement of nucleic acid sequences associated with the therapeutic gene and the gene itself comprise a unit called the expression cassette. The components of the expression cassette can include a promoter of gene expression, an enhancer of gene expression, localization (or secretion) signal, polyadenylation signal, Kosac sequence for efficient translation initiation, and RNA splicing signals.

Promoters and enhancers: constitutive versus inducible expression and tissue specificity. Mammalian genes contain non-coding DNA sequences that are promoters (or suppresors) of gene expression. These promoter sequences are located immediately upstream (5′) of the coding sequence (82). Promoters are influenced by soluble (trans-acting) factors that control DNA transcription. Enhancers influence the level and context of gene transcription from a remote location (82). Constitutively (continuously) expressed housekeeping genes are always turned on by their promoters and enhancers since the proteins they encoded are continuously required for normal cellular activities. Examples of housekeeping genes are cellular structural proteins (e.g., α-actin) and proteins involved in glycolysis (cell energetics). Inducible promoters activate gene transcription in response to ligand binding. The ligands that bind promoters often are called trans-acting factors. Examples of inducible promoters are: the metallothionein promoter which initiates gene transcription in the presence of certain divalent cations; glucocorticoid responsive element, which responds to corticosteroids; Lac-I promoter, which responds to the sugar lactose or the compound isopropylthiogalactoside (IPTG); heat shock promoter (HSP-70) induced by increased environmental temperature; and tetracycline-on promoter (tet-on), which responds to this class of antibiotics. Repressors terminate gene expression in the presence of a specific ligand, for example the tetracycline-off promoter (tet-off). Table 5 summarizes some promoters that differ in tissue specificity and inducibility.

Table 5. Examples of promoters of gene expression

Promoter	Tissue specificity of expression	Constitutive (C) vs. Inducible (I) expression
Antibody	B lymphocytes	C (low level) or I
Glucocorticoid	Many	C (low level) or I
Globin	Bone marrow and muscle	C
α-Fetoprotein	Yolk sac, liver, GI	C
HSP-70	Many	I
Metallothionein	Liver	C (low level) or I
L7	Cerebellum and retina	C
Locus control regions (Hb, CD2, MHC)	Tissue specific	C or I
Gonadotropin	Pituitary gland	C (low level) or I
Myosin	Muscle	C
Elastase	Exocrine pancreas	C
α-Crystalline	Eye lens	C
c-Ha-ras	Exocrine pancreas	C
Insulin	Endocrine pancreas	C (low level) or I
RSV-LTR	Many	C
Adenovirus MLP	Many	C
Cytomegalovirus late promoter	Many	C
SMCMHC	Smooth muscle cells	C
Tet-on	Many	I (tetracycline induced)
Tet-off	Many	I (tetracycline repressed)

HSP-70 = Heat shock protein; RSV LTR = rous sarcoma virus long terminal repeats; MLP = Major late promoter; and SMCMHC = smooth muscle myosin heavy chain; tet = tetracycline.

Tissue specificity of gene expression (83) in the gene therapy setting is critical in some therapeutic applications and not in others. For example, delivery of the cystic fibrosis transmembrane regulator gene to submucosal glands of the tracheobronchial tree is required to alleviate much of the pulmonary symptomology of cystic fibrosis. In contrast, any cell secreting sufficient coagulation factor VIII or IX into the blood would alleviate the symptoms of hemophilia type A or B, respectively. By selection of an appropriate promoter for transgene expression, one can broadly deliver genetic material to many cell types, but selectively express the gene in the subpopulation of cells that has the appropriate trans-acting factor.

Other DNA sequences that influence gene expression. Matrix adhesion regions and scaffold attachment regions are DNA sequences that enhance gene expression (84). These DNA regions lead to interaction of the transgene, with the nuclear envelope enhancing transcription. Transcriptionally active genes tend to be associated with the nuclear envelope. The Kosac sequence is a short sequence of nucleotides found at the beginning of most mRNA transcripts (85). The Kosac sequence enhances the binding of ribosomal subunits to the messenger RNA made from a gene, increasing the rate of protein translation. Polyadenylation signals are required at the end of genes for the addition of multiple adenylate residues to the gene's mRNA. Polyadenylation of mRNA is essential for the transport of the message from the nuclear region of the cell to the cytoplasm for translation. Polyadenylation protects the message from rapid degradation within cells (82).

Introns, intervening non-coding DNA sequences, are found in all genes of eukaryotic organisms. Genes are divided into regions that code for the functional region of the protein product (exons) separated by introns. Most gene products are modular in configuration. Specific signals at the border of intron and exon regions trigger excision of the non-coding regions from the

mature mRNA prior to translation (82). This modular configuration of genes allows exon shuffling facilitating development of novel proteins without requiring a large sequence of unique DNA to be developed. In addition, the intron-exon blueprint allows alternate forms of a single protein to be made by differential splicing, adding or removing regions of the protein. Differential splicing is most obvious for proteins with two forms: one that is a secreted protein, and the other that is membrane bound, as is associated with some immunoglobulins (IgG and IgD). Addition of artificial intron-exon structure to transgenes has been documented to greatly enhance expression in transgenic animals.

Secretory signals are short sequences of amino acids that trigger the export of the protein out of the cell (86, 87). The secretory signal for antibodies works in concert with alternate mRNA splicing by incorporating or deleting a transmembrane-spanning anchor. The resultant two immunoglobulin forms are either membrane bound or released into the extracellular compartment. The nuclear localization signal informs the nuclear pore complex that a protein is to be transported within the nuclear envelope (88).

Selectable markers and reporter genes. A positive selectable marker gene codes for a protein that protects cells from deleterious effects of specific substances (selection agent) to which the cell will be exposed (89). The selection agent will kill cells in vivo or in vitro that do not contain the protein encoded by the selectable marker gene. The dihydrofolate reductase gene (DHFR) is a positive selectable marker when methotrexate is used as the selection agent in cell culture media (positive selection). Cells that have received the DHFR gene as a result of gene therapy will survive methotrexate exposure, allowing only genetically engineered cells to survive in culture for subsequent transplantation. The TK enzyme is a negative selectable marker previously discussed in the production of herpesvirus and vaccinia gene therapy vectors (89) where acyclovir or 5'-BrdU is the corresponding negative selection agent. Table 6 summarizes commonly used selectable markers, pharmacologic agents used for selection, and whether selection imparts cell survival (positive) or cell death (negative).

Reporter genes often are used to confirm that DNA transfection has occured by the production of a protein product that can be visualized or quantified (90). Reporter genes are incorporated to ascertain the efficiency of new DNA transfer method, test the specificity of a putative tissue specific promoter, examine the efficacy of new gene therapy vectors or methods, visualize tissue specific cell expression, and follow cell lineage. Commonly used reporter genes are listed in Table 7.

The transgene. The protein product encoded by the expression cassette has an important contribution to the overall hazard in gene therapy experiments. Regardless of whether there is collateral exposure to a gene therapy vector, the consequence of the exposure is the untoward expression of the protein product encoded by the delivered gene. In determining relative risk associated with inadvertent exposure to a gene therapy vector, one needs to consider the various classes of proteins that may be encoded: structural proteins, enzymes (including reporter genes and selectable markers), cell signaling proteins (cytokines, neurotransmitters), toxins, antisense messages, hormones, or dominant negative receptors.

The undesirable effects resulting from expression of structural proteins would be limited to the particular cells that had been phenotypically altered by gene delivery. This is in contrast to the widespread or systemic effects that might occur from expression of cell signaling ligands or toxins. The potential deliterious effects of expressing enzymes are dependent on the availability of substrate, product of enzymatic activity, and location of the enzyme (intra- or extracellular). However, expression of cytokines or peptide hormones could lead to more global body system influences.

Table 6. Selectable markers in expression cassettes

Gene	Product	Selection agent	Selection
NeoR	Aminoglycoside phosphotransferase	G418	+
Hyg	Hygromycin B phosphotransferase	Hygromycin	+
DHFR	Dihyrofolate reductase	Methotrexate	+
TK	Thymidine kinase	5'BrdU or Gancyclovir	-
HGPRT	Hypoxanthine-guanine phosphoribosyl transferase	HAT	+
CD	Cytosine deaminase	5'-Fluorocytosine	-
AD	Adenosine deaminase	9-β-D-xylofuranosyl	+
ZeocinR	Bleomycin phosphotransferase	Bleomycin	+

+ = positive selection, - = negative selection.
HAT = hypoxanthine, aminopterin, thymidine medium.

Biosafety considerations in experiments using viral gene therapy vectors need to include the potential outcome of inadvertent gene transfer to personnel outside the realm of the proposed experiment. Given that the intent of gene therapy is to enhance (or down-regulate) protein expression, the consequences of the expression of the gene product should be a paramount biosafety concern. Genes encoding proteins that lead to systemic effects when expressed in small quantities (e.g., toxins, peptide hormones, cytokines, growth factors) need particular attention during risk assessment.

Immune competence of the host. The time course over which a transgene is expressed can be markedly prolonged when the vector also causes expression of viral proteins that make the transfected cell a target for immune system attack. Immune suppression in animals by use of cyclophosphamide and of immune-deficient rodent models allows adenovirus vectors to continue to express their protein product on the order of weeks, instead of days. The ablation of $CD4^+$ lymphocytes or gamma interferon is sufficient to prevent the elimination of adenovirus-transfected hepatocytes in the face of a pronounced cytotoxic lymphocyte response (38).

Many of the expression cassettes for gene therapy are constructed using bacterial cloning systems. The mammalian immune system has evolved mechanisms to recognize and attack cells that contain unmethylated CpG dinucleotide (cytosine and guanosine) motifs since these are found in bacterial DNA (91). The innate immune response is orchestrated by macrophages, dendritic cells, and natural killer cells which, on recognition of the CpG motif, release cytokines that activate T lymphocytes. The Th1 inflammatory responses are commonly associated with gene therapy, and can be induced by use of naked DNA oligonucleotides with CpG motifs.

Gene vector shedding. Passive shedding of replication-deficient adenoviral gene vectors in urine in laboratory animals and humans for up to 48 h after intravascular or intravisceral administration during in vivo gene therapy has been documented. The presence of replication-deficient adenoviral vectors for one week in the pericardial fluid (the duration of a catheter-based

Table 7. Reporter genes

Gene	Substrate	Product
Enhanced fluorescent proteins (EGFP, EYFP, DsRed, ECFP)	Ultraviolet light	Green, yellow, red, cyanin fluorescence
Chloramphenicol acetyltransferase	Tritiated-chloramphenicol	[^{3}H]chloramphenicol
Luciferase	Luciferin, ATP	Visible light
β-galactosidase	X-gal	Blue reaction product

epicardial delivery experiment) also has been documented (92). In animals without pre-existing immunity to vaccinia virus, shedding of recombinant vaccinia virus occurs after subcutaneous and intradermal inoculations (32, 48, 66). Transient viremia could be detected for up to 48 h in the sera of macaques after intramuscular inoculation with recombinant AAV; however, infective particles were not detected in biological excreta (28).

The pattern of secretion and excretion of viral vectors is influenced by vector titer, vector type, route of administration, and immune status of the recipient. On the basis of the few viral vector shedding studies published, the secretions and excretions from subjects receiving replication-deficient viral vectors should be considered biohazardous for 72 h after inoculation (29). Vaccinia virus-inoculated animals should be treated as potentially hazardous while skin lesions are apparent after inoculation, up to 14 days.

Conclusion

Each component risk of a gene therapy experiment contributes to the overall experimental risk. To recapitulate, component risks include the gene vector (viral or non-viral, viral replication-deficient or replication-competent), vector tropism, stable or transient transgene expression, methods of transgene delivery (ex vivo or in vivo) and potential for aerosol exposure, level and context of transgene expression (derived from noncoding DNA sequences), and immune status of the recipient. The gene therapy experiment with the greatest conceptual risk would be that which uses a replication-competent viral vector that stably transfects a constitutively expressed toxin transgene in all cells transfected in an immune deficient host (Table 2). Conversely, the experiment with the lowest conceptual risk is one that uses a non-viral vector to deliver a reporter transgene, the expression of which is induced transiently at low levels in a specific cell population of an immune competent host. Additional precautionary practices beyond those ordinarily used at animal biosafety level (ABSL-) 2 might be warranted if the untoward expression of the transgene would induce illness in personnel that are inadvertently exposed to the vector. Most gene therapy experiments in laboratory animals, using viral vectors, are carried out at ABSL-2, with additional concerns associated with the transgene sometimes leading to the incorporation of ABSL-3 practices (known as ABSL2+). Gene therapy experiments using non-viral vectors are generally carried out at ABSL-1, addressing additional concerns regarding the transgene by incorporating ABSL-2 practices.

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