29
Regulatory Aspects in the Development of Gene Therapies

Rosemarie Aurigemma, Joseph E. Tomaszewski, Sheryl Ruppel, Stephen Creekmore, and Edward A. Sausville

1. INTRODUCTION

Preclinical therapeutics development research is directed toward fulfilling two overlapping sets of goals. A set of scientific goals includes defining the best molecule or biologic construct for the task at hand, and proving the case for its development. The second set of goals addresses regulatory requirements necessary to introduce the agent into human subjects. In the case of "small molecule" drugs, in most cases the identity of the molecule and appropriate safety studies are straightforward. In contrast, the development of biologic agents, including gene therapies discussed here, presents distinct challenges. The nature of the "drug" may be an organism subject to mutation or selection of variants through recombination. Its properties may vary depending on the scale and method of its preparation, purification, and storage. How to test adequately for its safety prior to first introduction in humans may not be straightforward owing to intrinsic differences in response to the agent expected in humans as compared to animals.

The general principles, however, in allowing "first-in-human" experiences are similar for both small molecules and biologics. The ethical conduct of clinical trials in patients with a dire or life-threatening disease demands an understanding of the identity and dose of an agent that has the possibility of causing clinical benefit with adverse events expected at worst to be easily reversible and well predicted by the preclinical experience. In normal volunteers or patients who are otherwise well, evidence should be gathered that would support an initial range of doses of the test agent expected to be without substantial toxicity or long-term effects.

Thus, the successful clinical introduction of a novel therapeutic concept requires an organized approach to integrate scientific, technical, and regulatory requirements. This integration should begin in the research laboratory, as the concept becomes a candidate for the clinic, to prevent avoidable and expensive delays in clinical development. For example, if a product is created using a mammalian cell line for which viral or other contamination has not been ruled out, costly rederivation will be required before that product can be manufactured for clinical trials using current good manufacturing practices (cGMP). On the other hand, during the discovery phase, an excessive and premature concern over cGMP compliance can impede research. Therefore, a clear strategic understanding of the principles underlying regulatory issues is desirable and is the goal of this chapter.

We proceed from the experience of the Developmental Therapeutics Program (DTP) of the National Cancer Institute in the manufacture of biologicals, including gene therapy constructs for preclinical and clinical use. We outline the basis for our approach to safety testing studies to be included in an

From: *Contemporary Cancer Research*
Cancer Gene Therapy
Edited by: D. T. Curiel and J. T. Douglas © Humana Press Inc., Totowa, NJ

Table 1
Beyond a Good Idea: What the Successful Investigator Has Already Done With a Project Leading to Commercial Development

Defined candidate biologic (or molecule)
Made comparisons with similar products
Characteristics of product are consistent with pharmaceutical requirements
Production scale is adequate
Product characterization is adequate
Laboratory reference standard exists
In vitro potency assay has been developed
Stability studies develop confidence product is a "drug"
Reproducible model systems have confirmed in vivo activity with clinical product
Early animal work includes some toxicology
Scale-up requirements practical for initial clinical trials
In general, reflects experience and scientific maturity of investigator

Investigational New Drug (IND) application to the Food and Drug Administration (FDA). We focus on studies that would allow phase I and perhaps early phase II clinical trials. In 2002, DTP contributed to over 40 different cGMP biological projects. Most of these activities were selected competitively from applications received from academic researchers or from the intramural laboratories of the National Institutes of Health (NIH).

DTP products include viruses for vaccines or gene therapy, plasmids, monoclonal antibodies, recombinant proteins, synthetic peptides, natural product fermentations, and oligonucleotides. During the 2002 fiscal year, over 30 different lots were manufactured and released under cGMP for clinical use or further cGMP manufacturing. Most products are destined for phase I or phase II clinical trials in cancer. Beyond early (phase I/II) clinical trials, technology transfer for some products has occurred, with further development through phase III now addressed by pharmaceutical companies.

Based on experience accumulated over several years, we abstracted the initial profiles of the more successful concepts (Table 1), as well as some early project characteristics that can impede clinical development (Table 2). We note correlations between the thoroughness of the early research, attention to "the rules," outlined in the references cited here, and the development of commercial interest in the product.

2. FDA/RECOMBINANT DNA ADVISORY COMMITTEE REGULATIONS REGARDING GENE THERAPY

2.1. Brief History

Gene therapy and other biologic therapeutics are regulated within the FDA by the Center for Biologics Evaluation and Research (CBER), which was created in 1972 to address products emerging from the new biotechnology. Reorganization at the FDA is currently under way that will result in regulation of many biotherapeutics by the Center for Drug Evaluation and Research, which has oversight of small molecule drugs. It is anticipated, however, that blood products, vaccines, and gene therapy products will remain with CBER. The Biological Response Modifiers Advisory Committee is a chartered advisory group with the role of advising the FDA to ensure the safety and effectiveness of biological products, including gene therapy. The Recombinant DNA Advisory Committee (RAC) also oversees gene therapy research through the NIH Office of Biotechnology Activities. The RAC was established in 1974 in response to public concerns regarding the safety of recombinant deoxyribonucleic acid (DNA) technology. Human gene transfer trials in which NIH funding is involved (either directly or indirectly) are to be submitted to the RAC for review.

Table 2
Issues Requiring Attention at the Outset of a Project

Issues
Inappropriate antibiotic selection markers (e.g., ampicillin for recombinant proteins)
Lab-scale affinity purification
Solubility problems
Low yield
Errors in genetic sequence
Extraneous genetic material
Poorly defined production systems
Inadequate purification schemes
Unvalidated or nonexistent in vitro potency assay(s)
Lack of key reagents (e.g., antibodies to desired product)
Poor biochemical characterization
Inappropriate raw materials
Raw material qualification problems
Inappropriate cell banks
Difficult or unidentified toxicology systems
Failed vendor qualification
Intellectual property concerns

In addition to the US agencies that develop the regulations that govern drug development and licensing, the International Conference on Harmonization (ICH) was formed in April 1990 involving the United States, the European Union, and Japan to address the issue of globalizing such regulations. The ICH Steering Committee meets at least twice a year to continue their agenda of updating and harmonizing regulations for medicinal products; they emphasize safety, quality, and efficacy. Expert Working Groups were formed within ICH to address specific topics related to these basic areas. Although the FDA has not formally adopted all of the ICH guidelines, these guidelines should be followed when they exist in preliminary form. For investigators planning to conduct investigational drug trials in foreign countries, it is imperative that they be familiar with, and adhere to, the regulations set forth by ICH.

2.2. *Current FDA and ICH Safety Guidance Statements*

In 1996–2001, a series of FDA and ICH guidance documents on characterization and preclinical safety evaluation of biotechnology-derived pharmaceuticals was developed *(1–8)*. These guidances represent the FDA's current thinking on preclinical safety evaluation of biotechnology-derived pharmaceuticals. These are defined as products derived from characterized cells using a variety of expression systems, including bacteria, yeast, insect, plant, and mammalian cells. The active substances may include proteins and peptides, their derivatives, and products of which they are components. These materials could be derived from cell cultures or produced using recombinant DNA technology, including production by transgenic plants and animals. Examples include cytokines, enzymes, fusion proteins, growth factors, hormones, monoclonal antibodies, plasminogen activators, recombinant plasma factors, and receptors. The intended indications for use in humans may include in vivo diagnostic, therapeutic, or prophylactic uses. The principles outlined in these guidance documents may also be applicable to recombinant DNA protein vaccines, chemically synthesized peptides, plasma-derived products, endogenous proteins extracted from human tissue, and oligonucleotide drugs.

The FDA defines gene therapy as "a medical intervention based on modification of the genetic material of living cells" *(9)*. Cells may be modified ex vivo for subsequent administration to humans or may be altered in vivo by gene therapy given directly to the patient. When the genetic manipulation is performed ex vivo on cells that are then administered to the patient, this is also considered a form of somatic cell therapy *(9)*. "The genetic manipulation may be intended to have a therapeutic or prophylactic

effect or may provide a way of marking cells for later identification. Recombinant DNA materials used to transfer genetic material for such therapy are considered components of gene therapy and as such are subject to regulatory oversight".

Specific information related to gene therapy issues is contained in the 1998 "FDA Guidance for Industry: Guidance for Human Somatic Cell Therapy and Gene Therapy" *(9)*. This guidance document updates and replaces the 1991 FDA "Points to Consider" on this subject *(9a)*. New information was intended to provide manufacturers with current information regarding regulatory concerns for production, quality control testing, and administration of recombinant vectors for gene therapy and of preclinical testing of both cellular therapies and vectors. The FDA defines somatic cell therapy as "the administration to humans of autologous, allogeneic, or xenogeneic living non-germline cells, other than transfusable blood products, for therapeutic, diagnostic, or preventive purposes."

3. PRECLINICAL SAFETY TESTING OF BIOLOGICALS

The evaluation of the safety of gene therapy products is perhaps one of the more difficult tasks that faces toxicologists in the drug development arena today. Because many of the agents, like other biologicals, are species specific and because these agents integrate into the host genome, the choice of animal models and study designs is fraught with uncertainty, and each product frequently breaks new regulatory ground. Until recently, many investigators working in this field were probably lulled into a false sense of security because of the close scrutiny that preclinical studies and clinical protocols received from the NIH RAC and the FDA. With the death of Jesse Gelsinger, a patient enrolled in a gene therapy clinical trial to correct a metabolic disease, in 1999 and the recent reports of a leukemialike disease produced in children who received gene therapy treatments to correct severe combined immunodeficiency disease (SCID) *(10–13)*, the safety of these agents is called into question more than ever.

As a result, the toxicologist is under even more pressure to design more rigorous safety evaluation programs. There have been a number of reviews in this area in recent years by toxicologists from the FDA *(14)*, industry *(15,16)*, and international workshops *(17)* that cover many of the fundamentals regarding safety evaluation of gene therapy products. These resources, in conjunction with this chapter and the various guidance documents from the FDA and the ICH *(7,9,18)*, can be used by toxicologists to develop sound safety programs. These issues are discussed in greater detail in the latter half of this chapter.

4. WHERE TO FIND REGULATORY INFORMATION

The basic foundation of regulations for drug development can be found in the Code of Federal Regulations, Title 21 Food and Drugs (21 CFR; *19-26*). In addition to Title 21, FDA maintains an extensive number of Web sites containing regulatory information that should be consulted during the development of a novel biotherapeutic. The collection of available regulatory information includes Points to Consider, Guidance Documents, Drafts, and reports from public forums and symposia as well as information on the meetings of the Biological Response Modifiers Advisory Committee. ICH also sponsors a Web site for obtaining the most recent guidelines. A free subscription to an e-mail advisory update service is also available (Table 3).

In addition to the regulatory guidelines provided by the FDA, the NIH has published, and frequently updates, the NIH Guidelines for Research Involving Recombinant DNA Molecules, which can also be found electronically (Table 3). Although published documents disseminated by the FDA and NIH are essential starting points for planning a cGMP development strategy, it is important to realize that, in this rapidly evolving field, some requirements may be reflected in public comments or a growing consensus among industry long before they are formally adopted. Furthermore, it is not unusual during the development of a new biologic to have also developed alternatives to conventional practices that are based on sound scientific data and are then implemented after discussions with the FDA.

Table 3
Web Sites for Regulatory Guidance

General FDA: www.fda.gov
CBER: www.fda.gov/cber/index.html
CBER guidelines: www.fda.gov/cber/guidelines.htm
General ICH: www.ich.org
ICH guidances: www.fda.gov/cber/ich/ichguid.htm
NIH/RAC Web sites:
Office of Biotechnology Activities: www4.od.nih.gov/oba/
RAC: www4.od.nih.gov/oba/rac/aboutrdagt.htm
NIH guidelines: www4.od.nih.gov/oba/rac/guidelines/guidelines.html
CBER and FDA e-mail update service:
CBER: www.fda.gov/cber/pubinfo/elists.htm
FDA: www.fda.gov/emaillist.html

5. BASIC REGULATORY PROCESS

Product-specific factors can influence the regulatory requirements for an investigational agent. These issues should be explored in detail with the FDA in a pre-IND meeting at which the IND sponsor presents relevant preclinical data and manufacturing and animal safety testing to support the proposed approach to clinical development. The types of further studies pertinent to the particular agent can then be proposed, and input from the agency help shape the final development plan.

Interactions should take place with regulatory authorities at intervals that will facilitate the development of a product (Table 4). A key issue frequently not understood is that regulatory demands become more stringent as a product moves from phase I (safety), through phase II (activity), to phase III (comparative efficacy) trials and licensure *(6,27–31)*. This philosophy reflects the conscious effort not to stifle innovation in early phase clinical testing, but to ensure that, by the time registration-oriented late-stage trials are contemplated, issues related to production variability, assay, and assurance of safety are mature and well-substantiated because the results of such trials could be the basis for sale of the agent to the public.

Another factor that affects the level of regulatory compliance is the nature of the study population. Products manufactured for Phase I trials in healthy normal volunteers typically must meet much stricter requirements than those studied in patients with dire, life-threatening conditions (e.g., cancer or end-stage acquired immunodeficiency syndrome). As improved technology becomes available, requirements also tend to increase *(27–31)*.

5.1. Development of a Product

The level of regulatory compliance to be followed during different stages of development is dependent on the type of biologic product and the technology available for supporting its development. Assays, methods, and technologies for monoclonal antibody development *(32)*, for instance, are better defined than the techniques available for some of the new virus vectors that are emerging. Furthermore, new technologies to support product development are also constantly evolving. The number of specific viral contaminant tests required of cGMP human cell lines, for example, has increased steadily as new pathogens are identified and assays become available.

As new scientific knowledge accumulates, novel regulatory challenges can appear. The issue of transmissible spongiform encephalopathy, for example, has resulted in stringent requirements in raw material qualifications and traceability *(33)*. To minimize the impact of regulatory changes, careful record keeping of all processes and materials involved in deriving the product is highly recommended.

Table 4
Organization of Activities and Interactions With Regulatory Authorities During Development

Phase of development	Investigator activity	FDA activity	NIH activity
Early	Demonstrated proof of concept in vitro Beginning scale-up of production and purification studies Basic assays available for identity, purity, and potency		
Preclinical	Animal model for efficacy established Assays well developed for identity, purity, and potency Animal safety studies completed Production, purification methods ready for clinical manufacturing	Pre-IND meeting	RAC review
Clinical phase I	Initial dose finding and safety studies carried out in humans Continued refinement of production and purification methods Refinement of assays Validation of stability studies in progress	30 Day review of IND	
Clinical phase II	Dose finding, initial efficacy, and further safety studies in humans Production, purification methods well defined Assay validation in progress Validation of stability assays completed	End of phase II meeting Monitoring of safety data, clinical protocols and annual reports	Monitoring of safety data and clinical protocols
Clinical phase III	Comparative efficacy studies, continued collection of safety data Production scale-up and validation nearly complete Assay validation complete BLA preparation in progress	Pre-BLA submission meeting Meetings with investigators as needed	Meetings with investigators as needed
Licensing	BLA filing Continued collection of safety data	Pre-approval inspection Comprehensive, multidisciplinary review	

Finally, because of the availability of improved techniques for characterizing certain biologicals, the FDA is reorganizing its regulatory approach in ways that are analogous to the regulation of small molecule drugs. Technical demands will rise as regulatory requirements become more standardized.

5.2. General Principles of Regulatory Requirements for a Well-Characterized Product

Beyond identification and confirmation of an interesting novel concept, a major challenge in the preclinical development of biologicals is the optimal allocation of research and development resources. Key to this is proper assessment of a candidate concept's readiness for clinical development. All applicants for the National Cancer Institute's biologicals production resources now receive a list of "generic questions" corresponding to the appropriate product type. At the beginning of a project, it is not always reasonable to expect all issues to be resolved, but the assumption is that, for a successful candidate, these issues should be in hand by the time the project is completing phase I clinical testing. Table 5 lists the generic questions for cGMP production of recombinant virus vectors.

Because it is not possible to provide a complete guide to cGMP development in a few pages, we highlight some concerns common to many projects arising from academic laboratories. These are based on DTP's experience (both successes and failures) with projects making the transition from the preclinical research phase to pilot clinical studies. Our discussion is organized primarily around the concepts of identity, purity, potency, and safety that underlie development, manufacture, and release.

5.2.1. Identity

From the viewpoint of regulatory compliance, it is essential to establish the identity of the product and the components used to generate it during manufacturing *(9,22)*. We have noted that, frequently in proposed gene therapy or recombinant DNA-derived products submitted to us, DNA sequencing shows some deviation from the sequence published and/or submitted by the investigator, sometimes with major consequences for the project. When the DNA product, such as plasmid vaccines, will be administered to the patient, full plasmid sequencing has occasionally revealed unacceptable genetic sequences outside the open reading frame, as passengers from previous experiments, spurious promoters, frame shifts resulting in translation of nonsense sequences beyond the intended termination, and so on. DNA sequencing and repair are available at relatively low cost compared to the cost of repeating critical preclinical experiments. The FDA now requires complete sequencing of vectors of sizes up to 40 kilobases (kb) *(34)*.

For viral vectors, genetic stability is a major concern, particularly with respect to the possible issues of recombination with generation of replication-competent viruses. Specific guidelines are provided for adenovirus, retrovirus, and lentivirus vectors *(9,35,36)*. For other virus vectors, specific assays (e.g., neurovirulence testing of recombinant poliovirus and herpes virus vectors) are required to ensure that an attenuated phenotype is preserved after scale-up. If possible, the investigator should attempt to assess the genetic stability of the vector during preclinical studies, after administration in vivo or propagation in vitro.

In addition to the gene therapy product itself, the cells used to produce the product must be similarly identified and qualified for cGMP manufacturing. Excellent guidance documents are available for the production of master cell banks, working cell banks, and master virus banks *(9,32,37)*. At minimum, the complete cell history should be known and documented, and the cells should be tested to verify their origin.

Peptide sequencing or mapping employing liquid chromatography-mass spectrometry is typically used to provide critical information for synthetic peptides and recombinant proteins. For recombinant vectors containing transgenes, the expression of the desired gene product should be verified, for example, by immunoassay using a specific antibody against the product.

Table 5
Generic Questions for Candidate Projects Involving Recombinant Virus Vectors

1. What amount of delivered product is desired? How are these quantities justified?
   A. Non-GMP for additional preclinical development
   B. cGMP (clinical grade)
2. Provide details of molecular construct(s), including starting materials (e.g., plasmid, relevant vector maps, detailed vector construction scheme, and so on)
3. Does the construct contain an antibiotic resistance gene or other selectable marker? Are alternative methods of selection available? Why was the proposed selection chosen?
4. Is the vector replication competent or replication defective? (For replication-selective vectors, what is the molecular basis of the selectivity and the conditions under which the vector would replicate?)
5. Does the vector have an altered cell tropism? Define. What is the effect of altered tropism on anticipated host toxicity?
6. Has this construct been sequenced? Provide a sequence in an electronic format.
7. Are data available evaluating the genetic stability of the recombinant vector? Have mutation rates been established and/or rates of reversion to either wild-type or alternate viral genomes?
8. Are data available evaluating the potential for genetic recombination with other organisms in the patient or in the environment?
9. Is the organism currently grown in a qualified cGMP cell line? If not, is there a qualified cell line available for propagation of this vector? Was the cell line genetically modified to support this vector? Provide details of its construction and any information regarding the stability of the genetic alteration in the cell line.
10. Is there a cGMP-qualified virus seed bank?
11. Provide details of the proposed production method.
12. Has this material ever been produced for laboratory or clinical studies using this production system?
13. Has this material been produced in a related or other production system? If so, please provide the details.
14. Please provide details of existing purification methods.
15. What is the average yield of the production system before and after purification? What is the largest amount of material that you have produced in your laboratory in a single production batch? Please provide average ratios obtained by this production method for virus particle/infectious unit and infectious units/cell. How does this scale to anticipated quantities for clinical trial?
16. How much material is available as a reference standard?
17. Is material available as bulk biological substance for preliminary pharmacology and toxicology studies?
18. Are there reproducible assays for the product? Please provide the following assays, if available:
    A. Identity
    B. Purity
    C. Potency
19. What are to be the release criteria for the product? How does one know that a lot of product is qualified for use?
20. In what form (lyophilized, formulated product, and so on) and fill size is the desired final product? What is the desired final product formulation?
21. Are there issues of formulation that must be resolved?
22. What is known about the product stability with respect to physical integrity and activity?
23. Do you have any information regarding the estimated costs of this production project?
24. Have you identified any possible sources of production with any commercial firms? Please provide details.
25. Are there any safety issues connected with the production, purification, and/or handling of the product?
26. What is the status of the product(s) regarding intellectual property issues?
27. Sometimes, proposed projects are an improvement or modification of an existing approach. In these cases, this information may significantly affect the analysis of feasibility, cost, and other production issues. Please provide a brief summary of the nature of any such antecedents or other approaches that appear closely related to the proposed project.
28. Have there been any meetings scheduled with regulatory agencies, such as a pre-IND meeting with the FDA or a presentation to the NIH RAC? If so, please indicate the type of meeting, the regulatory agency, and the date or proposed date.
29. If you have had a pre-IND or RAC meeting, were any issues concerning manufacturing, safety, or stability raised that will have an impact on producing your product?
30. Who will sponsor the IND for the proposed study?
31. Has a source of funding been identified for performing the clinical trial with this product?

5.2.2. Purity

Purification strategies depend on the nature of the biologic agent and expected impurities. These approaches are guided by the early development of reliable analytical techniques appropriate to the product and to the manufacturing approach. For example, purification of recombinant proteins and monoclonal antibodies for cGMP manufacture typically involve large-scale chromatography based on multiple isolation principles (e.g., charge, hydrophobicity, size, and so on). Specific contaminants, such as DNA, endotoxin, viruses from mammalian cell production systems, contaminants introduced in the manufacturing process, and the like must be quantified and may require additional specific purification measures to remove or inactivate them.

Problems in refolding or solubility, tendencies to aggregate, and product stability at intermediate holding points can be significant issues in process development for scale-up. These represent key challenges in scale-up from investigator laboratory-generated lots to a potentially suitable scale to allow clinical testing. Additional concerns include subtle degradations of proteins that can lead to undesirable immunogenicity. A major concern is the impact of each additional step on the downstream product, which should be reassessed using in vitro potency assays as well as physicochemical characterization. At major development milestones, selected in vivo models should be reexamined using purified product.

Production cells must be cGMP qualified and tested for adventitious agents and other contaminants, before initiation of production as well as at end of production. A number of cGMP-qualified cell lines and starting vectors are available commercially at relatively low cost and should be considered for use as raw materials to initiate cGMP seed banks in preference to shared materials of uncertain provenance despite the good intentions of the original provider. In the handling of cell lines, care should also be taken to avoid contamination (e.g., from media components, trypsin, or activities taking place in nearby laboratory space). Postproduction cells can be tested for specific contaminants in the presence of a viral product (e.g., using polymerase chain reaction [PCR]). In the presence of virus product, however, it is unlikely that the full set of cGMP tests (e.g., cocultivation) for adventitious agents can be performed on postproduction cells. Before initiating cGMP production, therefore, investigators should consider the parallel propagation of a mock-infected control to provide a surrogate postproduction test article.

In addition to the usual tests for sterility and purity of purified investigational product *(9,20,21)*, it is important to have an assay for residual host cell DNA. Assays for host cell proteins are not always required for all phase I products, but are required for phase II and beyond. This consideration is another reason to start with cGMP-qualified cell lines from a commercial source because host cell DNA and protein assays may already be available.

The general requirement for adventitious agent testing is given in guidance documents (e.g., ref. *9*). It is important to note that some specific assays are not yet described in published FDA documents, but can enter widespread cGMP practice by sponsor-based industry consensus, liability considerations, or other factors. Endotoxin assays are available as kits, which are useful to guide laboratory process development. A qualified good laboratory practice (GLP) laboratory, however, should perform endotoxin assays for clinical product release. Specific assays may also be required to quantify process residuals from production and purification components (CsCl, antibiotics, and so on). In production facilities, particularly those where different types of gene therapy products may be produced, assays are necessary to support decontamination and cleaning, product changeover, environmental monitoring, and raw material qualification *(25,38)*. In general, all equipment that has contact with the investigational product should be verified free of contaminants before use.

Special consideration must also be given to assays to qualify virus seeds and end product for the presence of defective particles, replication-competent viruses, or defective genomes. In addition to monitoring for replication-competent and/or pathogenic vectors during manufacture, suitable assays may also be required to monitor patients receiving the therapeutic agent. In this case, levels of sensitivity for detection must be suitable for different types of patient specimens (serum, urine, sputum, and

so on). Evolving requirements for long-term follow-up of gene therapy patients *(39)* should be consulted to ensure that proper assay support is maintained beyond the duration of the planned clinical trial. The FDA regulations governing the performance of assays that support the production of biologics for human use can be found in 21 CFR 211, subpart I, “Laboratory Controls” *(25)*. GLP regulations only per-tain to the performance of preclinical animal and in vitro studies *(23)*.

5.2.3. Potency

The measured activity of a biological candidate depends on the hypothesized molecular mechanism of therapeutic action (21 CFR 600.3; *9, 19*). Although it is most reassuring to see in vivo demonstration of efficacy in appropriate animal models, the efficient development of a cGMP process will strongly benefit from the availability of rapid, reliable, and reproducible in vitro assays relating to the mechanism of action in addition to assays for purity and identity. Assays based on the basic therapeutic mechanism, therefore, are critical goals of early research and development.

5.2.4. Formulation and Stability

Formulation development should begin as early as possible as suitable assays become available and experience with real-time stability accumulates. It is preferable to choose formulations from candidates likely to be acceptable to the FDA, such as those whose components are already used for licensed products. As production reaches larger scales, handling and storage considerations become increasingly important. Stability studies incorporate assays for identity, purity (including aggregation), and potency. Although they can provide some useful information, accelerated stability studies are typically not reliable for predicting real-time stability of biologics. Therefore, there is a need for real-time stability studies to be initiated as soon as possible.

Suitability of formulated product should also be assessed in the identical administration and handling conditions expected in the clinic. This may include transient exposure to conditions expected during transit to the study site and storage in an environment that closely mimics study site conditions. These may result in markedly different product behavior at the study site from that expected from the behavior of vouchered specimens at a central repository site. The results of ongoing stability studies are useful to support process development; to evaluate product at intermediate hold points in scale-up production and at product release; and to support formulation development, product storage, shipment, and handling during toxicology studies and clinical applications. As development proceeds, master specifications for release of intermediate and final product should be established and refined. The IND must indicate a schedule for real-time stability studies to be performed throughout the duration of the clinical trial.

6. ORGANIZATION OF RESEARCH ACTIVITIES TO OPTIMIZE DEVELOPMENT OPPORTUNITIES

Some key early milestones common to all product areas include the attainment of an adequate scale of high-quality, single-batch production, the availability of adequate amounts of high-quality laboratory reference standard, and the development of reliable assays for identity, purity, and potency of the product. These milestones are necessary in addition to the exploration of animal models showing safety and promising evidence of efficacy. At early stages in a project, investigators should expect substantial variability in product quality, assays, and animal models.

Ideally, therefore, a single high-quality batch should be used to establish laboratory standards, support multiple assay qualification runs, and perform replicate animal model experiments. Multiple production runs could then be performed to explore process development issues, including scale-up. In this way, fundamental issues could be explored at the research stage to prepare for development required for cGMP manufacture. Following this reasoning, our facility often manufactures high-quality GLP lots to provide a uniform supply of material for additional preclinical research and development for selected biologics of interest before making the decision to undertake cGMP production.

6.1. Good Laboratory Practices

The early establishment of certain aspects of GLP (21 CFR 58) is crucial to the advancement of a drug development project. By following simple rules of laboratory cleanliness, documentation, and segregation of materials and activities at the start of development, time can be saved by avoiding the necessity to duplicate results that were not properly performed or documented from the outset.

At the discovery phase, development of reliable assays to explore basic therapeutic mechanisms of action are just as important as the performance of animal models in laying the groundwork for future cGMP product development. Laboratory facilities and staff should be adequate to perform necessary studies. Assay protocols should be specific and reproducible. Research documentation should be kept at a GLP level with complete and secure laboratory notebooks. Records of all reagents (i.e., manufacturer, catalog number, lot number, Certificates of Analysis [COAs], and expiration dates) should be routinely archived. Even if cGMP production or testing is not contemplated in the development laboratory, fluctuations in product activity are not unusual during later scale-up, and these materials and information may be useful in resolving such issues. Key assays for product or reagent identity should be repeated at appropriate intervals.

6.2. Segregation of Laboratory Activities

Access to critical raw materials and reagents, reference standards, and cell banks should be limited. Staff should avoid comingling of research-grade, GLP, and GMP activities or reagents by labeling reagent containers and sequestering them as much as possible. Similarly, signs should be posted on dedicated equipment, and access should be limited as appropriate. If common equipment must be used, standard operating procedures should be developed to define the use and control of such equipment, to clean equipment before and after use to avoid cross-contamination, and to document the use, cleaning, and calibration of the equipment.

6.3. Raw Materials

Critical raw materials (e.g., those used in seed development or pilot product manufacture) must be traceable to their source and obtained from reliable vendors. It is beneficial when possible to use vendors subjected to commercial audits. Animal-derived reagents should be avoided; reagents such as glycerol, detergents, proteins, amino acids, and the like should preferably come from vegetable sources. If this is not possible, animal-derived reagents should come from acceptable herds in countries without endemic or questionable transmissible spongiform encephalopathy *(33)*. It is important to ensure that raw materials are stored under appropriate conditions and not used beyond their expiration date. Inventories and logbooks should be used to track use of important reagents.

Critical materials requiring special storage conditions should be stored at more than one location to prevent loss in the event of equipment failure. cGMP-qualified cell lines should be purchased from vendors if possible, but if cGMP-qualified cell lines do not exist, cell lines should be obtained directly from a reliable repository source such as the American Type Culture Collection (ATCC) and documentation should be archived. Incoming cell lines must be tested for sterility and mycoplasma contamination. Thorough records should be kept on cell passages, observations, frozen storage, and the COAs from media and other components used to grow, freeze, and otherwise manipulate the cells. Critical cell lines should be segregated to prevent cross-contamination. Stock cells should not be cultured in incubators containing virus-infected cell lines.

Vectors should be purchased from a reliable vendor, and documentation should be kept on the propagation, storage, and use, including COAs, lot numbers, and so on of the reagents used to propagate the vector. If the vector is acquired from another laboratory (i.e., is unavailable for purchase from a vendor), a detailed history should be obtained of the generation of the vector, and detailed records should be kept from that time. All genetic manipulations of the vectors should be well documented and verified by sequencing.

6.4. Reference Standards

A lab-generated reference standard is a critical raw material for a biologic. It is ideal to have a large enough stock of this reference standard to use for the duration of the development work. It is often not possible, however, to produce sufficient quantities or material of sufficient stability at the early development stages. For this reason, it may be necessary to produce fresh batches and to test them thoroughly against independent standards or the current standard before that standard is depleted or loses potency.

The same consideration should be given to other critical reagents, such as cell lines and compounds obtained from outside sources. Reference standards and key reagents should be made or obtained in adequate amounts, characterized as well as possible, and stored under conditions that will maintain stability for at least the duration of the development process. As improved manufacturing and assay processes are developed, improved reference standards will be required, but quantities of the original standards should also be preserved to provide material for later comparisons as required *(40)*. In some cases, such as for retrovirus and adenovirus vector development, the FDA has made available reference material against which all sponsors can standardize their own reference reagents.

6.5. Assays and Resources

To avoid future questions about data reliability, investigators should consider outsourcing of difficult but common technologies, such as transmission electron microscopy, tandem liquid chromatography mass spectroscopy, peptide mapping, or DNA sequencing, if these are not adequate in their facility. Product-specific assays, such as potency studies and pilot animal efficacy and toxicity studies, are likely to be performed best at the researcher's own facility, early in development. Preclinical assay protocol development and record keeping must ensure that data are useful for later IND submission. At some point in cGMP process development, consideration should be given to technology transfer of critical assays to a GLP-compliant laboratory prepared to support the repetitive studies required during cGMP process development, manufacture, release, and postrelease stability studies for the duration of the clinical trial.

6.6. Preparation of Toxicology Material

Toxicology material should ideally be manufactured using the same process used to manufacture the cGMP clinical material. Therefore, a toxicology lot is produced late in process development. If there are concerns over batch-to-batch variability, production of a single lot for both toxicology and the initial phase I trial is recommended. Typically, a toxicology lot can be available several months before the clinical lot is ready for release.

7. SPECIAL REGULATORY CONSIDERATIONS

7.1. Cellular Therapies

For studies involving autologous cells, the handling of cells must be under GMP conditions to preserve sterility and prevent cross-contamination with other cells. For allogeneic cells, it is important to use a cGMP-tested cell line with adequate traceability, including its origin, passage history, and exposure to media products that may have been derived from animal sources.

7.2. DNA Plasmids

Starting material must be routinely checked for sequence accuracy; therefore, the complete plasmid should be sequenced. It is also preferable to examine genetic stability that can lead to the introduction of coding errors and changes in protein expression. The use of penicillin-like antibiotics (β-lactams) for selection is unacceptable because of the possibility of allergic reactions in patients administered products produced using this selection system. Other antibiotics, such as kanamycin,

are substituted, or alternative methods of selection are employed. Measures of DNA quality include supercoiled content, as well as assays for endotoxin, genomic DNA, and other contaminants from the production system. More in-depth guidance is available through guidance documents *(9)*.

7.3. Viral Agents

It is generally recommended by the FDA that a vector smaller than 40 kb must be completely sequenced *(34)*. As technology improves, this criterion may well be expanded to include larger vector genomes. Those vectors with genomes larger than 40 kb (e.g., herpes viruses, poxviruses) must have the transgene sequenced along with 5' and 3' flanking regions and any significant modifications to the vector backbone or sites vulnerable to alteration during molecular manipulation.

When qualified vaccine strains exist for the vector of interest (e.g., vaccinia, poliovirus) it is preferable for cGMP manufacture to derive investigational constructs using the vaccine strain if available from the NIH, ATCC, or commercial sources. For adenovirus vectors, the recent availability of an adenovirus reference standard allows for the normalization of dosing based on virus particle concentration and infectious unit (IU) titer. Current recommendations by the FDA are for a ratio of viral particle to infectious unit of less than or equal to 30:1 *(35)*.

For replication-defective adenoviruses, generation of replication-competent adenoviruses (RCA) must be measured in lots produced for clinical use. The current target requirement is fewer than 1 RCA per 3×10^{10} virus particles as measured by a cell culture/cytopathic effect method *(35)*. For viruses that are replication selective, different testing strategies (e.g., quantitative PCR) may be called for and should be discussed with the FDA. Similarly, the agency may have special considerations for viruses with altered tropism to ensure appropriate containment and prevent the generation of a replication-competent adenovirus with an expanded cell tropism. It should be noted that RCA assays must be optimized regarding the presence of defective particles and other factors that may affect the sensitivity of the assay.

Retroviruses are of special concern because of the possibility of insertional carcinogenesis. This potential safety problem is amplified if replication-competent retroviruses (RCRs) are generated *(9,36)*. The general guideline is to test at least 5% of the total virus vector supernatant produced by amplifying any RCR on a permissive cell line. In addition, 1% of the producer cells or 10^8 (whichever is less) must also be tested at the end of production by the method of coculturing on permissive cells *(36)*. As with adenovirus vectors, retrovirus vectors with tropism modifications are of special concern and may require more stringent containment and patient follow-up *(39)*. Promoter modifications may also affect the safety profile of these virus vectors.

Lentiviruses generally have the same safety concerns as retroviruses, particularly because they can replicate in a broader variety of cells (dormant as well as actively dividing cells). Although there is a retrovirus standard available through ATCC to investigators who are developing retrovirus vectors, there is no lentivirus standard currently offered. A lentivirus standard is not planned for the future primarily because of the great variability in lentivirus backbones currently under development for clinical investigations (e.g., equine, murine, human). Herpes viruses under development for clinical use either must be replication defective or, if replication competent, must be shown to be nonneurovirulent. Neurovirulence is an issue for poliovirus as well. Adeno-associated virus (AAV) vectors are of concern because, although these vectors are designed to be maintained episomally, there can be reversion to wild type, resulting in integration into the host chromosome, or the vector could be rescued in a patient with a concurrent adenovirus infection *(41)*.

Several interesting concepts seek to employ modified bacteria as the therapeutic agent. As with recombinant viruses, general issues of safety as well as specific issues of genetic stability and exchange should be considered. Stabilization of the new genetic material may be required by incorporation into the bacterial genome rather than through a plasmid that can be lost or exchanged. Strategies to incorporate new genetic material into bacterial DNA will depend on confirming the sequence accuracy of the target bacterial sequences as well as the novel genetic material. Introduction of an antibiotic resis-

tance gene through a manufacturing step raises special concerns and can be avoided using alternative selection approaches.

8. SAFETY, TOXICOLOGY, AND BIODISTRIBUTION STUDIES

8.1. Safety Study Design Issues

8.1.1. General Toxicity Issues

Whether evaluating small molecules or biologically derived materials such as gene therapy products, the basic intent of nonclinical toxicity studies is to define the pharmacological and toxicological effects predictive of the human response, not only prior to initiation of phase I clinical trials in humans, but also throughout the entire drug development process leading ultimately to Biologics License Application (BLA). The goals of these studies include, first, to define an initial safe starting dose and dose escalation schemes for first-in-human clinical trials; second, to identify potential target organs for toxicity, biomarkers or other parameters that can be monitored in patients receiving these therapies, and to determine if this toxicity is reversible; and finally, to determine which patient populations may be at greater risk for developing toxicity to a given cellular or gene therapeutic product *(42)*.

These nonclinical studies should be designed with the following points in mind: whether the product is transduced cells, the population of cells to be administered, or the class of vector used; the most appropriate animal species and physiological state of that model most relevant for the clinical indication and product class; and the intended doses, route of administration, and treatment regimens that will be used in the clinic. Many of the questions that need to be taken into consideration and addressed during the design phase for safety studies include what is already known about the most likely toxicities related to the agent's biodistribution, local as well as systemic toxicity, immune responses (immunogenicity and immunotoxicity), the potential for insertional mutagenesis, and biological activities of the transgene product. Then specific questions that arise with the new product or use are addressed. For example, are the safety issues primarily related to the vector, the transgene product, the method of administration, the formulation/excipient, or some combination of the above? How might existing published or unpublished nonclinical or clinical data address the questions mentioned above? Safety issues that should be addressed in these studies include evaluation of the toxicity of the vector alone (irrespective of the transgene), including its potential toxicity and/or tumorigenicity (in some cases, this is apparent from previous evaluations with the same vector); toxicity of transgene expression in vivo that may not be evident from in vitro studies; occurrence and consequences of ectopic transgene expression in nontargeted tissues; occurrence and consequences of immune responses to transgene or vector proteins such as autoimmunity; and finally the possibility of germline transduction *(34)*.

Because conventional pharmacology and toxicity testing as typically used for the evaluation of small molecules may not always be appropriate to determine the safety and biologic activity of cellular and gene therapy products, issues such as species specificity of the transduced gene, permissiveness for infection by viral vectors, and comparative animal to human physiology should be considered in the design of these studies. Available animal models mimicking the disease indication may be useful in obtaining both sufficient safety and efficacy data prior to entry of these agents into clinical trials. Early pre-IND discussions with the FDA during development of a toxicology plan may prevent delays and added expenses because of inadequate data or the use of inappropriate species. Some of the questions that should be answered by preclinical pharmacology/toxicology studies are the following *(43)*: What is the relationship of the dose to the biologic activity? What is the relationship of the dose to the toxicity? Does the route and/or schedule affect activity and/or toxicity? What risks can be identified for the clinical trial?

For ex vivo gene transfer, the product is considered to be the transduced cells. The general safety test (21 CFR 610.11) must be performed on the final product. When appropriate, modified procedures may be developed according to 21 CFR 610.9. The FDA is considering proposed rule making to amend the general safety test rules and scope of applicability, especially for cell therapy products *(9)*.

Finally, it is expected that these nonclinical toxicity studies will be conducted in compliance with GLP regulations. However, there will be situations in which highly specialized assays will be required because of the nature of biotechnology-derived products, and it will not be possible to conduct these assays in full compliance with GLPs (e.g., in university or other discovery laboratories). It will be important that these areas be identified for any impact that they may have on the interpretation of toxicity data. In most cases, carefully performed studies such as this can be used to support INDs and BLAs *(7)*.

8.1.2. Animal Model/Species Selection

When selecting the animal model that will be used in the various preclinical biodistribution, pharmacology, and toxicology studies, consideration should be given to the scientific rationale for the animal species used. For example, would there be an advantage to performing the studies in rodents when larger numbers of animals might be more practical, or is there a necessity for a large animal model, such as a canine or nonhuman primate? If nonhuman primate studies are proposed, is it clear that another large animal or rodent model would not provide the same information? Would there be any utility in a genetically deficient model, and would this deficient model be more relevant to the proposed study either because of the potential for adverse immunologic consequences or because of the biological effects in the deficient condition?

Animal models of disease may not be available for every cellular or gene therapy system proposed for development. This makes species selection an even more difficult process. Preclinical pharmacological and safety testing of these agents should employ the most appropriate, pharmacologically relevant animal model available. A relevant animal species might be one in which the biological response to the therapy mimics the human response. This entails some knowledge of the pathophysiology of the disease in humans and of how faithfully it is reproduced in the animal model.

The species of animal chosen for preclinical toxicity evaluations of viral preparations should be selected for its sensitivity to infection and production of pathologic sequelae induced by the wild-type virus related to the chosen vector, as well as its utility as a model of biologic activity of the vector construct. There should be a reasonable expectation of a similar distribution of receptors or permissivity in the animal model as there is in humans. Thus, the species utilized may vary with the vector administered, the transgene expressed, the route of administration, the patient population treated, and the disease studied. Rodent models rather than nonhuman primates may be useful if they are susceptible to pathology induced by the virus class (e.g., cotton rats are semipermissive hosts for adenovirus infections) *(44)*; the use of the SCID mouse *(45)* or the cotton rat *(46)* may be suitable for the evaluation of herpes simplex virus (HSV) rather than the *Aotus* monkey. Some investigators have also suggested the use of miniature swine for evaluation of adenoviral vectors *(47,48)*. When evaluating the activity of a vector in an animal model for the clinical indication, safety data can be gathered from the same model to assess the contribution of disease-related changes in physiology or underlying pathology to the response to the vector. Some specialized circumstances illustrating these points follow.

8.1.2.1. Cotton Rat Model and Adenovirus

The inbred cotton rat (*Sigmodon hispidus*) has been used extensively as an animal model for research since the 1940s, when it was first used in poliomyelitis research. Since that time, it has been shown to be a semipermissive host for adenoviral infection *(44,49)*. In those studies, it was shown that the pulmonary lesions and replication pattern of the virus seen in the cotton rat paralleled those seen in humans. Virus persisted in the nasal mucosa and lung for up to 21 and 28 days, respectively, after inoculation. This was even in the presence of high-titer neutralizing antibody that was detected by day 7.

Although cotton rats have readily adapted to the laboratory environment, they have retained a number of the characteristics of their wild counterparts. These animals have a tendency to bite, panic when handled, jump out of their cages, and have a large fight-or-flight zone. Care and handling of these animals have been described by other investigators *(50,51)*.

The cotton rat has been used for the evaluation of numerous adenovirus vectors by many routes of administration, and some of these studies are described here. When early E3-deleted adenoviral vectors were evaluated in the cotton rat, it was discovered that the E3 region was not required for replication, but that this region plays a critical role in the pathogenesis of the disease in that these mutants induced a markedly greater lymphocyte and macrophage/monocyte inflammatory response in the lungs *(52)*. E3 replacement recombinants were significantly less pathogenic than E3-deleted viruses after intranasal administration *(53)*. This study also demonstrated that adenovirus replicated in BALB/c and CBA mice and produced results that were similar to those seen in cotton rats.

The intracranial administration of a replication-defective adenovirus expressing the herpes simplex virus thymidine kinase (HSV*tk*) gene at a dose of 1.0×10^9 pfu into both adenoviral immune and adenoviral naïve cotton rats resulted in only mild gliosis and trace meningitis along the injection tract and approximated a "no toxic effect" dose *(54)*. When this same vector was administered to either Wistar rats or rhesus monkeys, direct neuronal injury or a dose-related inflammatory response was seen at the injection site and in the surrounding parenchyma. There was no apparent injury to tissues not of the central nervous system in any of the three models, and all cerebral spinal fluid, blood, urine, and stool samples failed to culture for adenovirus.

In a study with a similar HSV*tk* adenovirus inoculated into cotton rats via intracardiac injection at doses up to 3×10^{10} viral particles per animal with and without ganciclovir (GCV), the only significant microscopic lesions observed were epicardial inflammation and splenic hemosiderosis *(55)*. Vector sequences persisted throughout the 14-day assay period in the heart, lung, and lymphoid organs. Infectious virions were detected for 24 hours, but these virions were only detected at the site of injection of two animals in the highest dose group.

When a similar vector was administered as either one or two subcutaneous injection cycles with 2.3×10^{12} viral particles/kg each or as a single course with 6.9×10^{13} viral particles/kg, the only significant treatment-related histopathological finding was dermatitis with mild acanthosis at the site of vector injection *(56)*. In addition to these local effects, mild hyperamylasemia, lymphocytosis, and granulocytosis were seen clinically, but no other clinical signs of toxicity or death were observed. Vector sequences were detected in the skin at the injection site and to a lesser extent in the liver, spleen, and lungs, and small amounts of vector DNA were detected in the ovaries. These were cleared rapidly, and the absence of viral sequences in the excreta and swabs of the majority of animals suggested that there was no significant replication of this adenovirus vector in this host and little shedding.

8.1.2.2. *Aotus* Monkey Model and HSV

The owl monkey (*Aotus trivirgatus* or *nancymae*) has been an excellent model for oncogenic and nononcogenic viruses such as HSV type 1 (HSV-1) and others *(57)*, and the herpes virus that infects these animals is a strain of HSV-1 *(58)*. These animals have been routinely used to test vaccines against HSV-1 and found to mimic the course of the disease in humans *(59,60)*. As a result, it was only natural that these animals be used to evaluate the safety of gene therapy vectors produced from HSV-1. However, these animals tend to be more fragile to use than other species and as a result must be handled with greater care.

G207, an attenuated, replication-competent HSV-1 recombinant, was tested for safety after intracerebral inoculation in the *Aotus (61)*. These animals received doses of either 10^7 or 10^9 pfu of G207 or 10^3 pfu of the wild-type HSV-1 strain F. Wild-type HSV-1 caused rapid mortality and symptoms consistent with HSV encephalitis, including fever, hemiparesis, meningitis, and hemorrhage in the basal ganglia. For up to 1 year after G207 inoculation, seven of the treated animals were alive and exhibited no evidence of clinical complications, indicating that this form of HSV was considerably attenuated in comparison to wild-type virus. Two animals were reinoculated with 10^7 pfu of G207 at the same stereotactic coordinates 1 year after the initial dose. These animals were alive and healthy 2 years after the second inoculation.

As a further, more comprehensive clinical evaluation, animals were subjected to cerebral magnetic resonance imaging (MRI) studies both before and after G207 inoculation. These studies failed to reveal radiographic evidence of the typical HSV-related sequelae in the brain seen in the animals treated with the wild-type virus. Microscopic examination of multiple tissues found no evidence of HSV-induced histopathology or dissemination in spite of the fact that measurable increases in serum anti-HSV titers were detected. Viral shedding and biodistribution in the *Aotus* were also evaluated using PCR analyses and viral cultures of tear, saliva, or vaginal secretion samples *(62)*. Neither infectious virus nor viral DNA was detected at any time-point up to 1 month postinoculation. Analyses of tissues obtained at necropsy at 1 month or 2 years after inoculation showed the distribution of G207 DNA was restricted to the brain, although infectious virus was not isolated in these samples.

The safety of this construct was also evaluated in the *Aotus* after intraprostatic injections *(63)*. Safety was assessed on the basis of clinical observations, viral biodistribution, virus shedding, and histopathology. None of the injected monkeys displayed evidence of clinical disease, shedding of infectious virus, or spread of the virus into other organs. No significant microscopic abnormalities were observed in the organs evaluated. The results of these studies demonstrated that G207 can be safely inoculated into either the brain or the prostate, and that the *Aotus* monkey could be successfully used in preclinical toxicological evaluations.

In addition to the studies performed with this vector in *Aotus* monkeys, BALB/c mice were also used to evaluate the safety of G207. Mice were inoculated in the same manner as the *Aotus* either intracerebrally or intraventricularly with 10^7 PFU of G207 and survived for over 20 weeks with no apparent symptoms of disease. In contrast, over 80% of animals inoculated intracerebrally with 1.5×10^3 pfu of HSV-1 wild-type strain KOS and 50% of animals inoculated intraventricularly with 10^4 pfu of wild-type strain F died within 10 days. When mice were inoculated intrahepatically with G207 (3×10^7 pfu), all animals survived for over 10 weeks, whereas no animals survived for even 1 week after inoculation with 10^6 pfu of wild-type KOS *(64)*.

Mice were also injected in the prostate with either G207 or wild-type HSV-1 strain F and observed for 5 months *(63)*. None of the G207-injected animals exhibited any clinical signs of disease or died. However, 50% of mice injected with strain F displayed sluggishness and hunched behavior and were dead by day 13. On microscopic examination, the prostates injected with G207 were normal, whereas those injected with strain F showed epithelial flattening, sloughing, and stromal edema. These studies and those described by Whitley with the SCID mouse *(45)* point to the fact that rodents can be used in place of the owl monkey and produce adequate safety data for the evaluation of HSV-1 vectors for gene therapy.

Finally, safety data can also be obtained in well-designed efficacy studies. In many cases, mouse studies can provide similar information as studies conducted in nonhuman primates, so smaller species should not be automatically rejected. The nonhuman primate should not be relied on for use as a model simply because of the comfort of going into studies in humans only after evaluation of the toxicity of the agent in nonhuman primates. Experience has repeatedly shown for numerous classes of agents, both small molecules as well as biologicals, that no one species may be predictive of all human toxicities, and that not all human toxicities may be seen in other animal species *(65)*. Finally, certain human populations may not be predictive of all other human populations. This last fact makes predicting each and every toxicity almost impossible.

8.2. Dose, Route, and Schedule

The doses of vectors used in nonclinical studies should be selected based on preliminary efficacy/activity data from both in vitro and in vivo studies. A no-effect dose level, an overtly toxic dose, and several intermediate doses should be evaluated, along with appropriate controls, such as naïve or vehicle-treated animals. For new formulations, it is very important to include this last group to distinguish formulation-related effects from those of the agent of interest. When products are difficult to

produce in large quantities and as a result are in limited supply or for products with an inherently low toxicity, a maximum tolerated dose may not be achievable; as a result, a maximum feasible dose may be administered as the highest level tested in the preclinical studies and should be so designated in appropriate reports. Although this may not be intellectually or scientifically satisfying, the data derived from such a study should at least establish the safety of the clinical starting dose. Preclinical safety/toxicity studies should include at least one dose that is equivalent to and at least one dose escalation level exceeding those proposed for the clinical trial. The multiples of the human dose required to determine adequate safety margins may vary with each class of vector employed, and the relevance of the animal model to humans and the rationale for dose selection should be provided.

Scaling of doses based on either body weight or total body surface area as appropriate facilitates comparisons across the animal species used and humans. Although most small molecule cancer therapeutics are scaled based on body surface area *(66)*, body surface area may not be appropriate for gene therapeutics. Information generated in the preclinical studies can be used to determine the margin of safety of the vector for use in the clinical trial, as well as gage an acceptable dose escalation scheme depending on the steepness of the toxicity curve.

In a cross comparison of doses for an adenoviral vector for cystic fibrosis *(14)*, very similar toxicities were seen in cotton rats, mice, hamsters, rhesus monkeys, and baboons when the agent was directly instilled into the lungs of the animals. When the doses were scaled for body surface area, the no observable adverse effect levels for the various species were remarkably similar to one another and to the first human dose at which toxicity was observed, $0.4–2.4 \times 10^9$ IU/m^2 vs 1.2×10^9 IU/m^2 for humans. The only notable exception was the rhesus monkey at 4.6×10^7 IU/m^2. Studies like this enable other investigators to make wiser choices in the selection of doses and species to evaluate.

The route of administration of vectors can have an obvious influence on toxicity in vivo because of the distribution and concentrations of the agent that are produced. For example, intravenous bolus doses can produce very high concentrations for short durations; other routes of administration, such as subcutaneous, may produce much lower concentrations and more prolonged exposure. Current practice recommends that safety evaluations in preclinical studies should be conducted by the identical route and method of administration as that proposed for the phase I clinical trial whenever possible. When this is difficult to achieve in a small animal species, a method of administration similar to that planned for use in the clinic is advised. For example, intrapulmonary instillation of adenoviral vectors by intranasal administration in cotton rats or mice is an acceptable alternative to direct intrapulmonary administration through a bronchoscope because the latter procedure is simply not feasible in rodents. If the proposed clinical route is a nonintravenous (e.g., intratumoral), it may be wise also to conduct an intravenous study to provide perhaps "worse-case" data for what may happen in the event of an accidental injection directly into a patient's blood vessel.

When possible, the schedule of administration in the animal studies should also be identical to that intended in the phase I clinical trial. This may not be feasible in certain instances because of the production of neutralizing antibodies in the animal model that might preclude repeated administration; that may not be a factor in humans. In studies in which additional agents will be administered in combination with the gene therapy agent (e.g., in suicide therapy using HSV*tk* and GCV or HSV cytosine deaminase and 5-fluorocytosine), the route and schedule should also be identical to that planned for the clinic. Evaluating the vector alone in animal models would not provide sufficient data for predicting additional toxicity that may be produced by the combination, but should be at least one arm of the planned preclinical animal studies.

8.3. Pharmacological and Toxicological End Points

At a minimum, treated animals should be monitored for general health status (clinical observations, body weight and temperature changes, changes in food and water consumption), serum biochemistry, and hematological profiles. Target organs and other critical tissues should be examined for gross and microscopic changes. The addition of other parameters to be evaluated will depend on the nature

of the product studied, the species used, and the route of administration. There is no set of all-inclusive parameters that is sufficient for each and every new agent. Studies should be designed specifically for each agent, utilizing the most appropriate tests to capture as much relevant data as possible.

8.4. Immunogenicity

Because many biotechnology-derived pharmaceuticals intended for human use will be immunogenic in animals, the use of animal-derived proteins/products, if available, should be considered to define the intrinsic toxicity of the new agent. This may entail parallel development processes in which a construct relevant to the species in the safety test is developed to a point to allow a most relevant safety test to proceed. The analogous human construct then may actually be brought into the clinic supported by these results. If human material is used, measurement of antibodies associated with administration of products should always be performed when conducting repeated dose toxicity studies. These data will assist the investigator in the interpretation of the results of these studies.

Antibody responses produced in animals should be fully characterized (e.g., titer, number of responding animals, neutralizing or nonneutralizing), and their appearance should be correlated with any pharmacological and/or toxicological changes observed. More specifically, the effects of antibody formation on pharmacokinetics and/or pharmacodynamics, incidence and/or severity of adverse effects, complement activation, or the emergence of new toxic effects should be considered when interpreting the data. Attention should also be paid to the evaluation of possible pathological changes related to immune complex formation and deposition, especially in the kidney of treated animals.

The detection of antibodies in animals should not be the sole criterion for the early termination of a preclinical safety study or modification of the duration of the study unless the immune response neutralizes the pharmacological and/or toxicological effects of the biopharmaceutical in a large proportion of the animals. In most cases, the immune response to biopharmaceuticals in animals will be variable, similar to such responses in humans. If these issues do not compromise the interpretation of the data from the safety study, then no special significance should be ascribed to the antibody response.

The induction of antibody formation in animals is not necessarily predictive of a potential for antibody formation in humans. By the same token, humans may also develop serum antibodies against humanized proteins, and frequently the therapeutic response persists in their presence. The same may happen in animals if a purified protein is administered via a gene therapy viral vector. In the case of human factor IX, when the purified protein was administered to rhesus macaques, the monkeys did not make antibodies *(67)*. However, when factor IX was administered in a first-generation adenoviral vector, the animals mounted an acute phase response that produced neutralizing antibody that eliminated factor IX from the circulation *(68)*. Finally, the occurrence of severe anaphylactic responses to recombinant proteins is rare in humans. The results of guinea pig anaphylaxis tests, which are generally positive for protein products, are not considered predictive for reactions in humans; therefore, studies such as this are considered of little value for the routine evaluation of these types of products even though they are frequently performed.

Inflammatory, immune, or autoimmune responses induced by the gene product may be of concern. Animal studies should be conducted over a sufficient duration of time to allow development of such responses. Host immune responses against viral or transgene proteins may limit their usefulness for repeated administration in the clinic. The immune status of the intended recipients of a gene therapy should be considered in the risk–benefit analysis of a product, particularly for viral vectors. If exclusion of immunocompromised patients would unduly restrict a clinical protocol, immune-suppressed, genetically immunodeficient, or newborn animals may be used in preclinical studies to evaluate any potential safety risks.

8.5. Safety Pharmacology Studies

It is extremely important to investigate the potential for undesirable pharmacological activity in appropriate animal models and, when necessary, to incorporate particular monitoring for these activ-

ities in nonclinical toxicity studies and/or clinical studies. Safety pharmacology studies are designed to measure functional indices of potential toxicity. These indices may be investigated in separate studies or may be carefully incorporated into the design of nonclinical toxicity studies. The aim of these studies should be to reveal any functional effects on the major physiological systems of the body (e.g., cardiovascular, respiratory, renal, and central nervous systems) that will have a major impact on whether or how the agent is administered in the clinic. Some of these investigations may include the use of isolated organs or other test systems that do not involve the use of intact animals, such as the use of a perfused rabbit heart model for the evaluation of torsade de pointes and QT prolongation *(69,70)*. The results from all of these safety pharmacology studies may allow a mechanistically based explanation of specific organ toxicities, which should be considered carefully with respect to human use and intended indications. The use of additional biomarkers, exemplified by cardiac troponin T or I *(71,72)* for agents with potential cardiac toxicity, may be warranted in additional nonclinical animal studies and/or in clinical studies in humans.

Pharmacology studies can be divided into three main categories, depending on the nature of the effect: primary and secondary pharmacodynamic studies and safety pharmacology studies. Safety pharmacology studies are defined in the ICH guidance document (S7A) on this subject *(18)* "as those studies that investigate the potential undesirable pharmacodynamic effects of a substance on physiological functions in relation to exposure in the therapeutic range and above." This last point is particularly important in that these studies should be conducted at dose levels or serum concentrations that are therapeutic targets based on prior efficacy/activity studies. Simply conducting these studies at low doses does not provide much useful information or adequately assess the safety of the agent.

The objectives of these studies are to identify undesirable pharmacodynamic properties of a drug substance that may have relevance to its human safety and toxicity; to evaluate more fully adverse pharmacodynamic and/or pathophysiological effects of a drug substance that were previously observed in nonclinical toxicology and/or clinical studies; and to investigate the mechanism of action of the adverse pharmacodynamic effects that were either previously observed or suspected. The investigational plan developed to meet these objectives should be clearly identified and delineated by the drug development team.

For biotechnology-derived products that achieve highly specific receptor targeting, it is often sufficient to evaluate safety pharmacology end-points as a part of well-designed toxicology and/or pharmacodynamic studies; therefore, the need for separate safety pharmacology studies can be reduced or eliminated. For those bioproducts that represent a new therapeutic class and/or those products that do not achieve highly specific receptor targeting, a more extensive evaluation in separate safety pharmacology studies should be considered.

8.6. Biodistribution/Pharmacokinetic Studies

Biodistribution studies are generally performed for gene therapy products, and typical pharmacokinetic studies used for most types of drugs that measure serum or plasma levels, half-life, clearance, and the like are generally not performed. These preclinical animal biodistribution studies are designed to determine the distribution of the vector to sites other than the intended therapeutic site as an indicator of potential toxicity. The goals of these studies are generally twofold: determination of dissemination of the vector to the germline and distribution of vector to nontarget tissues. The first has been routinely accomplished by assaying total gonadal tissue. The second provides information on potential target organs of toxicity. Both may be addressed in the same preclinical study. Studies may use normal, intact animals or animal models of disease. The latter study may be more representative of the clinical setting.

Whenever possible, the intended route of administration should be employed, again with the consideration that groups of animals might also be treated intravenously as a worst-case scenario. Transfer of the gene to normal, surrounding, and distal tissues as well as the target site should be evaluated using the most sensitive detection methods possible, such as reverse transcriptase PCR, and should

include evaluation of gene persistence. When aberrant or unexpected localization is observed, additional studies should be conducted to determine whether the gene is expressed and whether its presence is associated with any pathologic effects.

Biodistribution studies may not be necessary for all new agents *(73)*. With "previously defined" vectors, if there is previous experience with a similar vector, route of administration, formulation, and schedule (e.g., adenovirus type 5 vectors), if the transgene product is considered "innocuous" if expressed ectopically, and when the size of the new vector is not essentially different, biodistribution aspects of the prior agent may be referenced. On the other hand, studies may not be postponed if a new class of vector is used (i.e., there is little or no previous experience; e.g., AAV, lentivirus, others); if there is a change in the formulation (i.e., lipid carrier instead of an aqueous formulation); if the route of administration is changed to an intentional systemic route from local administration of the "established" vector; and finally, if the transgene has the potential to induce toxicity if it is aberrantly expressed in nontarget organs.

As with toxicity studies, there are a number of factors that should be taken into consideration when designing vector biodistribution studies. Regarding species selection, nonhuman primates are not always needed. Rodents may be perfectly acceptable. The animal gender should reflect the intended patient population. At least 3–5 animals per sex and group should be used as a minimum. The use of smaller animals (i.e., mice or rats) allows the inclusion of larger numbers of animals and the easy evaluation of more time points. As in other studies, the following dose groups should be included when practical: controls, the maximally feasible/clinically relevant dose, and a lower dose for establishment of the no observable adverse effect level. The route of administration should mimic intended clinical route to the greatest extent possible. Regarding animal sacrifice and/or sampling time points, an early point that reflects peak vector transduction/expression should be included, as should a later time-point determined by intended clinical use and a time-point that should reflect clearance from the gonads and nontarget organs to determine persistence. The following tissues are generally recommended: peripheral blood; gonads; injection site; highly perfused organs (to assist in determination of toxicity) such as brain, liver, lung, kidneys, heart, spleen; other tissues based on toxicity/pathology as determined by transgene (e.g., bone marrow); and those based on the route of administration, such as draining, contralateral lymph nodes. The methodology used to detect the agent should detect a sequence of the vector DNA (or ribonucleic acid) that is unique to that product and should be appropriate to detect the vector sequence adequately in tissue samples from both preclinical animal studies and samples obtained during the initial clinical trials. Many of the points presented and discussed in this section are elaborated in publicly accessible FDA documents *(43,73)*.

8.7. Dissemination, Persistence, and Shedding

Shedding of viral vectors through the skin or in excreta is of obvious concern with highly infectious viruses. To measure the dissemination, persistence, and shedding of these vectors, multiple tissues (e.g., brain, heart, lungs, spleen, liver, kidneys, ovaries, and skin) as well as bodily fluids such as urine, feces, tears, saliva, vaginal secretions, and skin swabs are taken at multiple time-points throughout the study and analyzed by real-time quantitative PCR for the presence of vector sequences. If the vector sequences are rapidly cleared and viral sequences are absent in the excreta and swabs of the majority of animals, this suggests that there was no significant replication of the vector in the host *(56,62,74)*.

8.8. Single-Dose Toxicity Studies

Even if the intended clinical schedule involves repeated doses, single-dose studies may generate useful data to describe the relationship of dose to systemic and/or local toxicity and the steepness of the dose/toxicity curve. Data from these studies can be used to select doses for repeated-dose toxicity studies. Information on dose–response relationships may be gathered through the conduct of a single-dose toxicity study or as a component of pharmacology or animal model efficacy studies. The incorporation

of safety pharmacology parameters in the design of these studies should be considered, which will reduce the number of animals used, the amount of product required, and the number of studies that must be performed.

8.9. Repeated-Dose Toxicity Studies

The route and dosing regimen for these studies (e.g., daily vs intermittent dosing) should reflect the intended clinical use or exposure (e.g., once a week for 3 weeks, every other day, etc.). A recovery period should be included to determine the reversal or potential worsening of pharmacological/toxicological effects and/or the potential for delayed toxic effects. For biopharmaceuticals that induce prolonged pharmacological/toxicological effects, recovery group animals should be monitored until reversibility is demonstrated. This may not be fundamentally obvious at the outset of the study. The duration of repeated dose studies should be based on the intended duration of clinical exposure and disease indication. This duration of animal dosing has generally been 1–3 months for most biotechnology-derived pharmaceuticals, but this probably will not be the case for most gene therapy studies. However, in the case of life-threatening diseases such as cancer, longer term studies are generally not required to support phase I trials.

8.10. Immunotoxicity Studies

One aspect of immunotoxicological evaluation includes the assessment of potential immunogenicity as described in Section 8.4. Many biotechnology-derived pharmaceuticals are intended to stimulate or suppress the immune system and therefore may affect not only humoral, but also cell-mediated immunity. Inflammatory reactions at the injection site may be indicative of a stimulatory response. It is important, however, to recognize that simple injection trauma or specific toxic effects caused by the formulation vehicle may also result in toxic changes at the injection site. In addition, the expression of surface antigens on target cells may be altered, which has implications for autoimmune potential.

8.11. Reproductive/Teratology Studies

For conventional small molecule drugs, reproductive toxicity is usually assessed in rats and rabbits. The species specificity and potential immunogenicity of biologicals has led to the increased use of nonhuman primates for this purpose. The need for reproductive and developmental toxicity studies will depend on the product, clinical indication, and intended patient population. The specific study design and dosing schedule may be modified based on issues related to species specificity, immunogenicity, biological activity, and/or a long elimination half-life.

8.12. Germline Integration

The issue of germline integration has prompted considerable public discussion *(75)*. For gene therapy products directly administered to patients, the risk of vector transfer to germ cells should be seriously considered. Animal testicular or ovarian samples should be analyzed for vector sequences by the most sensitive method available. If a signal is detected in the gonads, further studies should be conducted to determine if the sequences are present in germ cells as opposed to stromal tissues; techniques used may include, but are not limited to, cell separations, *in situ* PCR, or other techniques. Semen samples for analysis can be collected from mature animals, including mice, by well-established methods *(76,77)* for determination of vector incorporation into germ cells. Evaluation of biodistribution to the gonads may not be needed prior to all phase I clinical trials, and this issue should be considered carefully in pre-IND meetings with the FDA. The Informed Consent Form should address the lack of data and the unknown risks.

8.13. Genotoxicity Studies

Genotoxicity studies, such as the Ames salmonella assay, the micronucleus test, and the mouse lymphoma assay, which are routinely conducted for small molecule pharmaceuticals, are not appli-

cable to biotechnology-derived pharmaceuticals, especially gene therapy products, and therefore are not needed. The administration of large quantities of peptides, proteins, or viruses may yield uninterpretable results. When there is cause for concern about the product, genotoxicity studies should be performed in available and relevant systems, including newly developed systems. The use of standard genotoxicity studies as indicated for assessing the genotoxic potential of process contaminants is not considered appropriate. If standard assays are performed for this purpose, the rationale should be provided.

8.14. Carcinogenicity Studies

Standard 2-year carcinogenicity bioassays in normal mice and rats are generally inappropriate for biotechnology-derived pharmaceuticals and probably more so for gene therapy products. This issue has received additional attention owing to the emergence of a lymphoproliferative syndrome in a potentially significant fraction of recipients of a vector designed to treat SCID syndrome *(10,11)*. This clinical result actually recapitulates to a certain degree toxicities anticipated from experience in animal models *(78)*. Thus, product-specific assessment of carcinogenic potential will still be needed for biopharmaceuticals, and studies utilized must be refined after consideration of the duration of anticipated clinical dosing, the patient population, or the biological activity of the product (e.g., retrovirus vectors, growth factors, immunosuppressive agents, etc.).

When there is a concern about carcinogenic potential, a variety of new approaches may be considered to evaluate this risk. When the potential to support or induce proliferation of transformed cells and clonal expansion leading to neoplasia is considered possible, the product should be evaluated with respect to receptor expression for the biopharmaceutical or for the transgene's expressed form in various malignant and normal human cells, especially those potentially relevant to the patient population under study. The ability of the biopharmaceutical to stimulate growth of normal and/or malignant cells expressing the relevant receptors should be determined. When in vitro studies such as this give cause for concern about carcinogenic potential, further studies in relevant animal models may be needed if these are available and relevant.

As stated in this section, when gene transfer agents must be evaluated, the standard rodent models (mice and rats) and the 2-year carcinogenicity bioassay are probably not appropriate. Daily administration of vector as is usually performed in these studies is not feasible; however, several of these vectors, including AAV, continue to express over the lifetime of the animal. The other factor that may be limiting is that the host immune response to the vector or to the transgene may either limit the toxicity, perhaps because of the development of neutralizing antibodies, or may have effects on tumor development. It will be necessary to consult with the FDA to develop product-specific studies on an individualized basis or to determine whether and which carcinogenicity studies are needed.

8.15. Local Tolerance Studies

Local tolerance to administration of the new agent should be evaluated. The formulation intended for the clinical trial should be tested unless there is a cogent reason why this would not be feasible or biologically meaningful. In most cases, the potential adverse effects of the product at the site of administration can be evaluated in the single- or repeated-dose toxicity studies that are usually conducted in the normal course of development, thus eliminating the need for separate studies.

9. SPECIAL SAFETY CONSIDERATIONS PERTAINING TO VIRAL AGENTS

9.1. Adenovirus

Adenoviral vectors can efficiently deliver genes to a wide variety of dividing and nondividing cell types both in vitro and in vivo, resulting in a high level of transient gene expression. Considerable modifications have been made in the wild-type virus to reduce infectivity and toxicity in normal tissues or to improve transduction or tropism for tumor cells. The death of Jesse Gelsinger because of

several complications, including liver failure, coupled with the fact that adenovirus infections in immunocompromised oncology patients can lead to fatal hepatotoxicity *(79,80)*, and reports of serious hepatotoxicity and death in nonhuman primates treated with different adenoviral vectors make the safety evaluation of these vectors for cancer treatment paramount.

When a first-generation adenovirus vector expressing human factor IX was intravenously injected into rhesus macaques at doses from 1×10^{10} to 1×10^{11} pfu/kg, no toxicity was seen at the lower dose level, but substantial, dose-limiting liver toxicity was observed at the higher dose *(68)*. This hepatotoxicity was manifested as elevated serum transaminase levels, hyperbilirubinemia, hypoalbuminemia, and prolongation of clotting times. All evidence of liver toxicity resolved except for persistent hypofibrinogenemia in the high-dose recipient, indicating possible permanent liver damage. These data suggested a very narrow therapeutic window for this first-generation adenovirus-mediated gene transfer vector. In follow-up studies *(81)*, it was concluded that these abnormalities may be caused by direct toxic effects of the adenovirus vector itself, or may result indirectly from the accompanying acute inflammatory response marked by elevations in interleukin 6.

When another first-generation adenoviral vector expressing β-galactosidase was intravenously injected into two baboons at doses of 1.2×10^{12} or 1.2×10^{13} particles/kg, the baboon receiving the high dose developed acute symptoms, decreased platelet counts, and increased liver enzymes and became moribund at 48 hours after injection; the baboon receiving the lower dose developed no symptoms *(82)*. Again, a very narrow therapeutic index was demonstrated.

Recombinant adenoviruses infused into the portal vein of adult rhesus monkeys at a dose of 10^{13} particles/kg resulted in the formation of neutralizing antibody, severe liver toxicity, and death. Readministration of a second vector was associated with the same degree of toxicity as the first vector, but prompted a much more vigorous neutralizing antibody response *(83)*. The administration of several gene transfer vehicles and routes was studied in rhesus monkeys to develop a model for adenovirus-mediated gene transfer for liver. Vectors administered via the portal vein or saphenous vein were efficient, but this resulted in transient gene expression and was accompanied by an immune response to both vector and transgene products and acute hepatitis *(84)*.

Turning to models of intracerebral administration, baboons received intracerebral injections of either a high dose of a replication-defective adenoviral vector expressing HSV*tk* (1.5×10^9 pfu) with or without GCV or a low dose of ADV/RSV*tk* (7.5×10^7 pfu) with GCV to evaluate the safety of this regimen. Animals receiving the high-dose vector and GCV either died or became moribund and were sacrificed during the first 8 days of treatment. Necropsy of these animals revealed cavities of coagulative necrosis at the injection sites. Animals that received only the high-dose vector were clinically normal; however, lesions were detected with MRI at the injection sites corresponding to cystic cavities at necropsy. Animals that received the low-dose vector and GCV were clinically normal and exhibited small MRI abnormalities, and although no gross lesions were present at necropsy, microscopic foci of necrosis were present. Neutralizing antibodies were produced in the animals, but no shedding of the vector was found in urine, feces, or serum 7 days after intracerebral injection *(74)*.

Intrapulmonary administration uses are exemplified through the use of recombinant adenovirus vectors containing expression cassettes for human cystic fibrosis transmembrane conductance regulator, which were instilled through a bronchoscope into limited regions of lung in baboons. The only adverse effect noted was a mononuclear cell inflammatory response within the alveolar compartment of animals receiving doses of virus that were required to induce detectable gene expression. Minimal inflammation was seen at 10^7 and 10^8 pfu/mL, but at 10^9 and, more prominently, at 10^{10} pfu/mL, a perivascular lymphocytic and histiocytic infiltrate was seen *(85)*.

9.1.1. First-Generation Modified Adenoviral Vectors (E1- and E3-Deleted Vectors)

Host immune elimination of infected cells often limits gene expression in vivo to 1–2 weeks after infection *(86,87)*. In addition to a cell-mediated immune response to the adenovirus infection, a humoral

response to the injected virus is often generated *(88)*. Although this humoral response may prevent the use of adenoviral vectors for repeated dosing, it may be blocked or reduced by coadministration of immunosuppressive agents or cytokines. Alternatively, the use of adenoviruses of different serotypes may allow for repeated administration, even in the presence of neutralizing antibodies *(88)*.

Harvey et al. *(89)* reported on 6 years of experience with the local administration of low ($<10^9$ particle units) and intermediate (10^9 to 10^{11} particle units) doses of $E1^-/E3^-$ adenovirus vectors to six different sites. With a group incidence of only 0.7% for major adverse events and no deaths related to administration of the adenovirus vectors, local administration of low and intermediate doses of adenovirus vectors was well tolerated.

9.1.2. Second-Generation Modified Adenoviral Vectors (E1/E2- and E1/E4-Deleted Vectors)

Second-generation adenoviral vectors, mutated in E2a, have been proposed to decrease host immune responses against transduced cells, reduce toxicity, and increase duration of expression as compared with first-generation vectors deleted only in E1. The safety of and E1-, E2a-, E3-deleted adenoviral vector (Av3H82) encoding an epitope-tagged B-domain-deleted human factor VIII complementary DNA was evaluated in cynomolgus monkeys. Animals received intravenous administration of either 6×10^{11} or 3×10^{12} particles/kg. Vector distribution was widespread, with the highest levels observed in liver and spleen. Histopathology, hematology, and serum chemistry analysis identified the liver and blood as major sites of toxicity. Transient mild serum elevations of liver enzymes were observed, along with a dose-dependent inflammatory response in the liver. In addition, mild lymphoid hyperplasia was observed in the spleen. Mild anemia and a transient decrease in platelet count were observed, as was marrow hyperplasia and extramedullary hematopoiesis *(90)*.

When vectors deleted in E1 and containing either a temperature-sensitive mutation in the E2a gene or a deletion of the E4 region were infused into the hepatic artery of nonhuman primates, minimal toxicity was seen. Histopathology showed that portal inflammation was present throughout both livers in the animals receiving the high dose. No differences were seen in the level of portal inflammation in targeted and untargeted lobes. PCR analysis detected viral DNA sequence in gonads and brain as well as many other tissues in baboons treated with high-dose vector. In baboons treated with lower doses of an E1-E4-deleted vector expressing the human ornithine transcarbamylase gene, DNA was detectable by nested PCR in liver, but not gonads, at days 29 and 61. The data suggested that intraarterial administration of recombinant adenoviral E1-E4-deleted vector is feasible and safe. *(91)*.

Toxicity of first-generation and E2a-deleted vectors expressing human α1-antitrypsin was evaluated in C3H mice after administration of increasing doses starting at 1×10^{12} particles/kg. Both vectors induced dose-dependent toxicity, including transient thrombocytopenia, elevated alanine aminotransferase, and increased hepatocyte proliferation, followed by inflammation and then hypertrophy. There were no differences in toxicity between the two vectors when measured at matching levels of human α1-antitrypsin expression. However, the E2a-deleted vector had slightly reduced hepatocyte toxicity at an intermediate particle dose *(92)*. Although these vectors are purported to be less toxic, the fact remains that the human fatality that occurred in the ornithine transcarbamylase deficiency trial at the University of Pennsylvania was an E1, E4-deleted construct *(93)*.

9.1.3. Modified Tropism Adenoviruses

The current E1-deleted adenoviruses can infect a wide variety of cells through a specific interaction between the viral fiber protein and at least one cell surface receptor. Entry of the virus into the cell is further enhanced through a specific interaction of the fiber with an integrin "coreceptor." The host's range of tissue susceptibilities to the virus can therefore be altered by various strategies so that it can bind more efficiently to the target cell surface *(94–96)*. Antibodies against tissue-specific cell surface proteins can also be coupled to the fiber protein to facilitate partial targeting of the virus *(97)*. Another approach to achieve "targeting" of the virus is the use of cell-specific promoters to drive

expression of a therapeutic gene in the context of the recombinant virus *(98)*. Enhanced uptake strategies through fiber modification may present special concerns for toxicity, especially regarding hepatotoxicity when administered by an intravenous or direct hepatic artery injection. Careful comparison of a tropism-modified adenoviral vector to the nontropism-modified vector in mouse toxicity and biodistribution studies as well as nonhuman primate and toxicity studies might be desirable.

9.2. Adeno-Associated Viruses

Members of the family *Parvoviridae* AAVs are among the smallest of the DNA viruses *(99)*. Unlike autonomous parvoviruses, AAVs or dependo-viruses require coinfection with unrelated helper viruses for a productive infection to occur *(100,101)*. As recombinant vectors for gene therapy, they seem to have several advantages compared to other vectors, such as the transduction of terminally differentiated and nondividing cells *(102,103)*, relatively high stability of transgene expression *(104)*, and the potential for targeted integration *(105,106)*. From a safety point of view, AAV vectors show a lack of pathogenicity *(107–109)*, low immunogenicity *(104,110)*, and low risk of insertional mutagenesis *(111)*. Also, there did not appear to be any evidence of transduction in the gonads of rhesus monkeys *(112)*. However, AAV has a limited DNA capacity.

9.3. Herpes Simplex Virus

HSV vectors can deliver large amounts of exogenous DNA; however, cytotoxicity and maintenance of transgene expression are obvious obstacles to their use. They also have the advantages of the abilities to infect nondividing cells and to establish latency in some cell types. The ability to establish latency in neuronal cells makes HSV an attractive vector for treating neurological disorders such as Parkinson's and Alzheimer's diseases. In addition, the ability of HSV to infect efficiently a number of different cell types, such as muscle and liver, may make it an excellent vector for treating non-neurological diseases.

One problem associated with HSV-based vectors has been the toxicity of the vector in many different cell types. The generation of HSV vectors with deletions in many of the immediate early gene products, which is similar to the strategy used for adenovirus, has resulted in vectors with reduced toxicity and antigenicity as well as prolonged expression in vivo *(60–65)*.

No clinical study has been reported in detail with these vectors. Section 8.1.2. details a summary of preclinical safety considerations pertaining to use of the *Aotus* monkey in comparison to rodent species.

9.4. Retroviruses

Retrovirus vectors are replication-defective and are primarily based on the Moloney murine leukemia virus (MMLV), which is a well-studied and well-characterized retrovirus *(113,114)* with numerous advantages. They have been extensively studied, produce stable integration into the host genome, and are very efficient at gene transfer. Disadvantages include an infection that is limited to dividing cells, which makes gene transfer into nondividing cells such as hematopoietic stem cells, hepatocytes, myoblasts, and neurons an impossibility, and low titer of products.

There are four theoretical concerns that exist for retroviral-mediated gene transfer that relate to two potential delayed toxicities. These are insertional mutagenesis, recombination with endogenous retroviral sequences, transfer of exogenous genetic material, and accidental exposure to replication-competent murine retroviruses *(115)*. Because retroviral vectors can permanently integrate into the genome of the infected cell, there is a serious concern regarding insertional mutagenesis causing the development of a secondary malignancy. The presence of RCRs is of major concern because of the fact that RCRs have produced lethal malignant T-cell lymphomas in 3/10 rhesus monkeys *(78)*. These concerns resulted in a publication concerning the FDA considerations on these issues *(116)* and the issuance of a new FDA guidance on this subject in October 2000 *(36)*. Some of these con-

cerns are no longer theoretical. The elation that this type of retroviral-mediated therapy was successful in curing a number of children with SCID *(117)* has been severely dampened by the reports of a leukemialike disease produced in two of these children *(10–13)*.

9.5. Lentiviruses

Unlike oncoretrovirusus such as Moloney murine leukemia virus, one subclass of retroviruses, the lentiviruses, can infect nondividing cells. This makes these viruses attractive for gene transfer. One of these viruses, human immunodeficiency virus (HIV), has been the subject of investigation by a number of groups. The most obvious concerns with using HIV for gene therapy is safety and the possible generation of replication-competent virus during vector production. This involves engineering the vector so that it is replication defective. This has been done in a number of cases by eliminating all accessory genes, such as *tat, vif, vpr, vpu,* and *nef,* from a packaging construct that still has the ability to transduce cells *(118)*. Concern about the possibility of insertional activation of cellular oncogenes by a random integration of the vector provirus into the host genome has led to the development of self-inactivating vectors *(119–122)*. The use of self-inactivating viruses significantly improves the biosafety of HIV-derived vectors because it reduces the likelihood that RCRs will originate during vector production and target cells and hampers recombination with wild-type HIV in an infected host. In an attempt to make even safer constructs, other groups are working on the development of lentiviral vectors from HIV-2 *(123)*, simian immunodeficiency virus *(124)*, bovine immunodeficiency virus *(125,126)*, and feline immunodeficiency virus *(127)*. These last vectors may be inherently more acceptable because they are not based on HIV-1. None of these newer constructs has moved toward the clinic, so there is little animal safety data and no human data on these vectors yet.

10. SUMMARY AND CONCLUSIONS

This chapter presents a range of issues that might be considered in contemplating the development of a gene therapy agent to the point of an early phase clinical trial. As no gene therapy product has yet been recognized as "safe and effective," the standard approach to these issues should be regarded very much as a "work in progress." Indeed, the nature of these agents would suggest that each new opportunity would call for its own unique set of requirements, so that a single approach will probably never "standardly" exist. Rather, the principles that underlie regulatory policy should be woven into the approach to each new agent.

In broad strokes, these involve approaches to answering the following questions: Is the identity of the agent clearly defined? Can successive batches of the material be made reproducibly in the quantity to support clinical development, and how is this known? Are the biological features of the vector, and its transgene when applicable, clearly similar in the animal species used for safety studies and in the human, at least as far as this can be ascertained? What dose is likely to be required for therapeutic effect? What level of gene expression or replication is necessary to attain a therapeutic effect? When toxicity occurs because of the agent, what is the evidence the toxicity will be reversible? Is toxicity after repeated doses of agent likely to be attenuated or magnified by immunological response to the agent? What are the consequences of long-term presence of the therapeutic agent in the recipient? Is there a danger of producing directly (as the therapeutic agent itself) or indirectly (through recombination and/or replication) an infectious agent that acts horizontally in the population or vertically across generations? How will the presence and distribution of the gene therapy agent be followed in the patient?

Sponsors are above all encouraged to see the regulatory process as a collaborative interaction with the regulatory agencies with the end not only of protecting the patient, but also of advancing the most scientifically defensible and rigorous questions to clinical trial. Far more costly than the conduct of experiments designed to be compliant with regulatory requirements is a failed or overtly injurious clinical trial. A clear understanding and a proactive approach in addressing regulatory issues outlined

here will maximally ensure the likelihood of an interpretable clinical outcome. The regulatory issues outlined here must be approached with continuing appreciation of the evolving science associated with the gene therapy field. As such, requirements may evolve with the state of the science, and careful sustained contact with the regulatory agencies is important in incorporating the best and most current science into the design, conduct, and interpretation of regulatory studies.

REFERENCES*

1. ICH guidance on quality of biotechnology/biological products: derivation and characterization of cell substrates used for production of biotechnical/biological products, September 21, 1998. Available on-line at: http://www.fda.gov/cber/guidelines.htm.
2. ICH: final guideline on quality of biotechnological products: analysis of the expression construct in cells used for production of r-DNA derived protein products, February 1996. Available on-line at: http://www.fda.gov/cber/guidelines.htm.
3. ICH guidance on specifications: test procedures and acceptance criteria for biotechnological/biological products, August 18, 1999. Available on-line at: http://www.fda.gov/cber/guidelines.htm.
4. ICH Guidance for Industry Q1A (R2), stability testing of new drug substances and products, November 20, 2003. Available on-line at: http://www.fda.gov.cber/guidelines.htm.
5. ICH guidance on viral safety evaluation of biotechnology products derived from cell lines of human or animal origin, September 24, 1998. Available on-line at: http://www.fda.gov/cber/guidelines.htm.
6. ICH guidance: Q7A good manufacturing practice guidance for active pharmaceutical ingredients, September 25, 2001. Available on-line at: http://www.fda.gov/cber/guidelines.htm.
7. ICH guidance for industry: S6 preclinical safety evaluation of biotechnology-derived pharmaceuticals, July16, 1997. Available on-line at: http://www.ich.org.
8. ICH: final guidance on stability testing of biotechnological/biological products, July 10, 1996. Available on-line at: http://www.fda.gov/cber/guidelines.htm.
9. FDA guidance for industry: guidance for human somatic cell therapy and gene therapy, March 30, 1998. Available on-line at: http://www.fda.gov/cber/guidelines.htm.
10. Check, E. (2002) Gene therapy: a tragic setback. *Nature* **420,** 116–118.

10a. FDA Draft Points to Consider in Human Somatic Cell Therapy and Gene Therapy, August 27, 1991.

11. Hacein-Bey-Abina, S., von Kalle, C., Schmidt, M., et al. (2003) A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. *N. Engl. J. Med.* **347,** 255–256.
12. Check, E. (2002) Regulators split on gene therapy as patient shows signs of cancer. *Nature* **419,** 545–546.
13. Check, E. (2003) Second cancer case halts gene-therapy trials. *Nature* **421,** 305.
14. Pilaro, A. M. and Serabian, M. A. (1999) Preclinical development strategies for novel gene therapy products. *Toxicol. Pathol.* **27,** 4–7.
15. Pilling, A. M. (1999) The role of the toxicologic pathologist in the preclinical safety evaluation of biotechnology-derived pharmaceuticals. *Toxicol. Pathol.* **27,** 678–688.
16. Dempster, A. M. (2000) Nonclinical safety evaluation of biotechnologically derived pharmaceuticals. *Biotech. Annu. Rev.* **5,** 221–258.
17. Griffiths, S. A. and Lumley, C. E. (1998) Non-clinical safety studies for biotechnology-derived pharmaceuticals: conclusions from an international workshop. *Hum. Exp. Toxicol.* **17,** 63–83.
18. ICH guidance to industry: S7A safety pharmacology studies for human pharmaceuticals, July 12, 2001. Available at: http://www.fda.gov/cber/guidelines.htm.
19. Code of Federal Regulations, Title 21, Food and Drugs, Part 610.10, Subpart B, General biological products standards; general provisions; potency, p. 61, revised as of April 1, 2002.
20. Code of Federal Regulations, Title 21, Food and Drugs, Part 610.12, Subpart B, General biological products standards; general provisions; sterility, pp. 63–67, revised as of April 1, 2002.
21. Code of Federal Regulations, Title 21, Food and Drugs, Part 610.13, Subpart B, General biological products standards; general provisions; purity, pp. 67–68, revised as of April 1, 2002.
22. Code of Federal Regulations, Title 21, Food and Drugs, Part 610.14, Subpart B, General biological products standards; general provisions; identity, pp. 68–69, revised as of April 1, 2002.
23. Code of Federal Regulations, Title 21, Food and Drugs, Part 58, Good laboratory practice for nonclinical laboratory studies, pp. 302–316, revised as of April 1, 2002.
24. Code of Federal Regulations, Title 21, Food and Drugs, Part 210, Current good manufacturing practice in manufacturing, processing, packing, or holding of drugs; general, pp. 113–115, revised as of April 1, 2002.
25. Code of Federal Regulations, Title 21, Food and Drugs, Part 211, Current good manufacturing practice for finished pharmaceuticals, pp. 115–135, revised as of April 1, 2002.

*All websites accessed 05/17/2004.

26. Code of Federal Regulations, Title 21, Food and Drugs, Part 312, Investigational new drug application, pp. 57–95, revised as of April 1, 2002.
27. FDA guidance for industry: content and format of Investigational New Drug Applications (INDs) for phase I studies of drugs, including well-characterized, therapeutic, biotechnology-derived products, November 1995. Available on-line at: http://www.fda.gov/cder/guidance/phase1.pdf.
28. FDA guidance for industry: IND's for phases 2 and 3 studies of drugs, including specified therapeutic biotechnology-derived products, chemistry, manufacturing and controls content and format, April 20, 1999. Available on-line at: http://www.fda.gov/cber/guidelines.htm.
29. FDA guidance for industry: content and format of chemistry, manufacturing, and controls information and establishment description information for a vaccine or related product, January 5, 1999. Available on-line at: http://www.fda.gov/cber/guidelines.htm.
30. FDA guidance for industry for the submission of chemistry, manufacturing, and controls information for a therapeutic recombinant DNA-derived product or a monoclonal antibody product for in vivo use, August 1996. Available on-line at http://www.fda.gov/cber/guidelines.htm.
31. FDA guidance for industry: formal meetings with sponsors and applicants for PDUFA products, March 7, 2000. Available on-line at: http://www.fda.gov/cber/guidelines.htm.
32. FDA points to consider in the manufacturing and testing of monoclonal antibody products for human use, February 28, 1997. Available on-line at: http://www.fda.gov/cber/guidelines.htm.
33. FDA letter to manufacturers of biological products: recommendations regarding bovine spongiform encephalopathy (BSE), April 19, 2000. Available on-line at: http://www.fda.gov/cber/ltr/bse041900.htm.
34. FDA Biological Response Modifiers Advisory Committee: current policy on sequence characterization of gene transfer products, November 16, 2000. Available on-line at: http://www.fda.gov/cber.
35. FDA Biological Response Modifiers Advisory Committee: adenovirus titer measurements and RCA levels, July 13, 2001. Available on-line at: http://www.fda.gov/cber.
36. FDA guidance for industry: supplemental guidance on testing for replication competent retrovirus in retroviral vector based gene therapy products and during follow-up of patients in clinical trials using retroviral vectors, October 18, 2000. Available on-line at: http://www.fda.gov/cber/guidelines.htm.
37. FDA points to consider in the characterization of cell lines used to produce biologicals, May 17, 1993. Available on-line at: http://www.fda.gov/cber/guidelines.htm.
38. FDA Dear Gene Therapy IND or Master File Sponsor Letter, March 6, 2000. Available on-line at: http://www.fda.gov/cber/ltr/gt030600.htm
39. FDA gene therapy patient tracking system final document, June 27, 2002. Available on-line at: http://www.fda.gov/cber/genetherapy/gttrack.htm.
40. FDA guidance concerning demonstration of comparability of human biological products, including therapeutic biotechnology-derived products, April 1996. Available on-line at: http://www.fda.gov/cber/gdlns/comptest.txt.
41. Third national NIH gene transfer safety symposium: safety considerations in the use of AAV vectors in gene transfer clinical trials, March 7, 2001. Available on-line at: http://www4.od.nih.gov/oba/rac/transcript 3-7-011.pdf.
42. Cornetta, K. G. and Robertson, M. J. (2000) Basic principles of gene therapy: basic principles and safety considerations. In *Principles and Practice of the Biologic Therapy of Cancer*, 3rd ed. (Rosenberg, S. A., ed.), Lippincott, New York, pp. 733–747.
43. Pilaro, A. M. (2000, November 17) Preclinical animal models in gene therapy research. Biological Response Modifiers Advisory Committee Meeting. Available on-line at: http://www.fda.gov/cber/advisory/brm/brmmain.htm
44. Pacini, D. L., Dubovi, E. J., and Clyde, W. A. Jr. (1984) A new animal model for human respiratory tract disease due to adenovirus. *J. Infect. Dis.* **150,** 92–97.
45. Whitley, R. (2000, November 16) Use of *Aotus* monkey to assess neurovirulence of replication-selective herpes vectors. Biological Response Modifiers Advisory Committee Meeting. Available on-line at: http://www.fda.gov/cber/advisory/brm/brmmain.htm.
46. Lewandowski, G., Zimmerman, M. N., Denk, L. L., Porter, D. D., and Prince, G. A. (2002) Herpes simplex type 1 infects and establishes latency in the brain and trigeminal ganglia during primary infection of the lip in cotton rats and mice. *Arch. Virol.* **147,** 167–179.
47. Torres, J. M., Alonso, C., Ortega, A., Mittal, S., Graham, F., and Enjuanes, L. (1996) Tropism of human adenovirus type 5-based vectors in swine and their ability to protect against transmissible gastroenteritis coronavirus. *J. Virol.* **70,** 3770–3780.
48. Morrissey, R. E., Horvath C., Snyder, E. A., et al. (2002) Porcine toxicology studies of SCH 58500, an adenoviral vector for the p53 gene. *Toxicol. Sci.* **65,** 256–265.
49. Prince, G. A., Porter, D. D., Jenson, A. B., Horswood, R. L., Chanock, R. M., and Ginsberg, H. S. (1993) Pathogenesis of adenovirus type 5 pneumonia in cotton rats (*Sigmodon hispidus*). *J. Virol.* **67,** 101–111.
50. Prince, G. A. (1994) The cotton rat in biomedical research. *Animal Welfare Information Center Newslett.* 5. Available on-line at: http://www.nal.usda.gov/awic/newsletters/v5n2/5n2princ.htm.

51. Ward, L. E. (2001) Handling the cotton rat for research. *Lab. Anim.* **30,** 45–50.
52. Ginsberg, H. S., Lundholm-Beauchamp, U., Horswood, R. L., et al. (1989) Role of early region 3 (E3) in pathogenesis of adenovirus disease. *Proc. Natl. Acad. Sci. USA* **86,** 3823–3827.
53. Berencsi, K., Uri, A., Valyi-Nagy, T., et al. (1994) Early region 3-replacement adenovirus recombinants are less pathogenic in cotton rats and mice than early region 3-deleted viruses. *Lab. Invest.* **71,** 350–358.
54. Smith, J. G., Raper, S. E., Wheeldon, E. B., et al. (1997) Intracranial administration of adenovirus expressing HSV-TK in combination with ganciclovir produces a dose-dependent, self limiting inflammatory response. *Hum. Gene Ther.* **8,** 943–954.
55. Rojas-Martinez, A., Wyde, P. R., Montgomery, C. A., Chen, S. H., Woo, S. L., and Aguilar-Cordova, E. (1998) Distribution, persistency, toxicity, and lack of replication of an E1A-deficient adenoviral vector after intracardiac delivery in the cotton rat. *Cancer Gene Ther.* **5,** 365–370.
56. Wildner, O. and Morris, J. C. (2002) Subcutaneous administration of a replication-competent adenovirus expressing HSV-tk to cotton rats: dissemination, dersistence, shedding, and pathogenicity. *Hum. Gene Ther.* **13,** 101–112.
57. Barahona, H., Melendez, L. V., Hunt, R. D., and Daniel, M. D. (1976) The owl monkey (*Aotus trivirgatus*) as an animal model for viral diseases and oncologic studies. *Lab. Anim. Sci.* **26,** 1104–1112.
58. Leib, D. A., Hart, C. A., and McCarthy, K. (1987) Characterization of four herpesviruses isolated from owl monkeys and their comparison with *Herpesvirus saimiri* type 1 (*Herpesvirus tamarinus*) and herpes simplex virus type 1. *J. Comp. Pathol.* **97,** 159–169.
59. Meignier, B., Jourdier, T. M., Norrild, B., Pereira, L., and Roizman, B. (1987) Immunization of experimental animals with reconstituted glycoprotein mixtures of herpes simplex virus 1 and 2: protection against challenge with virulent virus. *J. Infect. Dis.* **155,** 921–930.
60. Meignier, B., Martin, B., Whitley, R. J., and Roizman, B. (1990) In vivo behavior of genetically engineered herpes simplex viruses R7017 and R7020. II. Studies in immunocompetent and immunosuppressed owl monkeys (*Aotus trivirgatus*). *J. Infect. Dis.* **162,** 313–321.
61. Hunter, W. D., Martuza, R. L., Feigenbaum, F., et al. (1999) Attenuated, replication-competent herpes simplex virus type 1 mutant G207: safety evaluation of intracerebral injection in nonhuman primates. *J. Virol.* **73,** 6319–6326.
62. Todo, T., Feigenbaum, F., Rabkin, S. D., et al. (2000) Viral shedding and biodistribution of G207, a multimutated, conditionally replicating herpes simplex virus type 1, after intracerebral inoculation in *Aotus. Mol. Ther.* **2,** 588–595.
63. Varghese, S., Newsome, J. T., Rabkin, S. D., et al. (2001) Preclinical safety evaluation of G207, a replication-competent herpes simplex virus type 1, inoculated intraprostatically in mice and nonhuman primates. *Hum. Gene Ther.* **12,** 999–1010.
64. Sundaresan, P., Hunter, W. D., Martuza, R. L., and Rabkin, S. D. (2000) Attenuated, replication-competent herpes simplex virus type 1 mutant G207: safety evaluation in mice. *J. Virol.* **74,** 3832–3841.
65. Olson, H., Betton, G., Robinson, D., et al. (2000) Concordance of the toxicity of pharmaceuticals in humans and in animals. *Regul. Toxicol. Pharmacol.* **32,** 56–67.
66. Freireich, E. J., Gehan, E. A., Rall, D. P., Schmidt, L. H., and Skipper, H. E. (1966) Quantitative comparison of toxicity of anticancer agents in mouse, rat, hamster, dog, monkey, and man. *Cancer Chemother. Rep.* **50,** 219–244.
67. Lozier, J. N., Metzger, M. E., Donahue, R. E., and Morgan, R. A. (1999) The rhesus macaque as an animal model for hemophilia B gene therapy. *Blood* **93,** 1875–1881.
68. Lozier, J. N., Metzger, M. E., Donahue, R. E., and Morgan, R. A. (1999) Adenovirus-mediated expression of human coagulation factor IX in the rhesus macaque is associated with dose-limiting toxicity. *Blood* **94,** 3968–3975.
69. Eckardt, L., Haverkamp, W., Borggrefe, M., and Breithardt, G. (1998) Experimental models of torsade de pointes. *Cardiovasc. Res.* **39,** 178–193.
70. Eckardt, L., Haverkamp, W., Mertens, H., et al. (1998) Drug-related torsade de pointes in the isolated rabbit heart: comparison of clofilium, *d,l*-sotalol and erythromycin. *J. Cardiovasc. Pharmacol.* **32,** 425–434.
71. Jaffe, A. S. (2001) Elevations in cardiac troponin measurements: false false-positives: the real truth. *Cardiovasc. Toxicol.* **1,** 87–92.
72. Sparano, J. A., Brown, D. L., and Wolff, A. C. (2002) Predicting cancer therapy-induced cardiotoxicity: the role of troponins and other markers. *Drug Saf.* **25,** 301–311.
73. Serabian, M. A. and Pilaro, A. M. (2000, November 17) Preclinical considerations for gene transfer clinical trials: vector biodistribution. Biological Response Modifiers Advisory Committee Meeting. Available on-line at: http://www.fda.gov/cber/advisory/brm/brmmain.htm.
74. Goodman, J. C., Trask, T. W., Chen, S. H., et al. (1996) Adenoviral-mediated thymidine kinase gene transfer into the primate brain followed by systemic ganciclovir: pathologic, radiologic, and molecular studies. *Hum. Gene Ther.* **7,** 1241–1250.
75. Recombinant DNA Advisory Committee (RAC), National Institutes of Health (1999, March 11–12). Meeting summary (available on-line at: www4.od.nih.gov/oba/rac/summaries/3-99sum.htm.) and meeting minutes RAC Minutes-03/11-12/99. Available on-line at: www4.od.nih.gov/oba/rac/minutes/3-99 RAC.htm.
76. Snell, G. D., Hummel, K. P., and Abelmann, W. H. (1944) A technique for the artificial insemination of mice. *Anat. Rec.* **90,** 243–253.

77. Kile, J. C. Jr. (1951) An improved method for the artificial insemination of mice. *Anat. Rec.* **109,** 109–117.
78. Donahue, R. E., Kessler, S. W., Bodine, D., et al. (1992) Helper virus induced T cell lymphoma in nonhuman primates after retroviral mediated gene transfer. *J. Exp. Med.* **176,** 1125–1135.
79. Zahradnik, J. M., Spencer, M. J., and Porter, D. D. (1980) Adenovirus infection in the immunocompromised patient. *Am. J. Med.* **68,** 725–732.
80. Haura, E. B., Winden, M. A., Proia, A. D., and Trotter, J. E. (2002) Fulminant hepatic failure due to disseminated adenovirus infection in a patient with chronic lymphocytic leukemia. *Cancer Control* **9,** 248–253.
81. Lozier, J. N., Csako, G., Mondoro, T. H., et al. (2002) Toxicity of a first-generation adenoviral vector in rhesus macaques. *Hum. Gene Ther.* **13,** 113–124.
82. Morral, N., O'Neal, W. K., Rice, K., et al. (2002) Lethal toxicity, severe endothelial injury, and a threshold effect with high doses of an adenoviral vector in baboons. *Hum. Gene Ther.* **13,** 143–154.
83. Nunes, F. A., Furth, E. E., Wilson, J. M., and Raper, S. E. (1999) Gene transfer into the liver of nonhuman primates with E1-deleted recombinant adenoviral vectors: safety of readministration. *Hum. Gene Ther.* **10,** 2515–2526.
84. Sullivan, D. E., Dash, S., Du, H., et al. (1997) Liver-directed gene transfer in non-human primates. *Hum. Gene Ther.* **8,** 1195–1206.
85. Simon, R. H., Engelhardt, J. F., Yang, Y., et al. (1993) Adenovirus-mediated transfer of the CFTR gene to lung of nonhuman primates: toxicity study. *Hum. Gene Ther.* **4,** 771–780.
86. Yang, Y., Su, Q., and Wilson, J. M. (1996) Role of viral antigens in destructive cellular immune responses to adenovirus vector-transduced cells in mouse lungs. *J. Virol.* **70,** 7209–7212.
87. Yang, Y. and Wilson, J. M. (1995) Clearance of adenovirus-infected hepatocytes by MHC class I-restricted $CD4^+$ CTLs in vivo. *J. Immunol.* **155,** 2564–2570.
88. Mack, C. A., Song, W. R., Carpenter, H., et al. (1997) Circumvention of anti-adenovirus neutralizing immunity by administration of an adenoviral vector of an alternate serotype. *Hum. Gene Ther.* **8,** 99–109.
89. Harvey, B. G., Maroni, J., O'Donoghue, K. A., et al. (2002) Safety of local delivery of low- and intermediate-dose adenovirus gene transfer vectors to individuals with a spectrum of morbid conditions. *Hum. Gene Ther.* **13,** 15–63.
90. Brann, T., Kayda, D., Lyons, R. M., et al. (1999) Adenoviral vector-mediated expression of physiologic levels of human factor VIII in nonhuman primates. *Hum. Gene Ther.* **10,** 299–301.
91. Raper, S. E., Haskal, Z. J., Ye, X., et al. (1998) Selective gene transfer into the liver of non-human primates with E1-deleted, E2A-defective, or E1–E4 deleted recombinant adenoviruses. *Hum. Gene Ther.* **9,** 671–679.
92. O'Neal, W. K., Zhou, H., Morral, N., et al. (1998) Toxicological comparison of E2a-deleted and first-generation adenoviral vectors expressing alpha1-antitrypsin after systemic delivery. *Hum. Gene Ther.* **9,** 1587–1598.
93. Batshaw, M. L., Wilson, J. M., Raper, S., Yudkoff, M., and Robinson, M. B. (1999) Recombinant adenovirus gene transfer in adults with partial ornithine transcarbamylase deficiency (OTCD). *Hum. Gene Ther.* **10,** 2419–2437.
94. Wickham, T. J., Carrion, M. E., and Kovesdi, I. (1995) Targeting of adenovirus penton base to new receptors through replacement of its RGD motif with other receptor-specific peptide motifs. *Gene Ther.* **2,** 750–756.
95. Krasnykh, V. N., Mikheeva, G. V., Douglas, J. T., and Curiel, D. T. (1996) Generation of recombinant adenovirus vectors with modified fibers for altering viral tropism. *J. Virol.* **70,** 6839–6846.
96. Miller, R. and Curiel, D. T. (1996) Towards the use of replicative adenoviral vectors for cancer gene therapy. *Gene Ther.* **3,** 557–559.
97. Wickham, T. J., Segal, D. M., Roelvink, P. W., et al. (1996) Targeted adenovirus gene transfer to endothelial and smooth muscle cells by using bispecific antibodies. *J. Virol.* **70,** 6831–6838.
98. Kochanek, S., Clemens, P. R., Mitani, K., Chen, H. H., Chan, S., and Caskey, C. T. (1996) A new adenoviral vector: replacement of all viral coding sequences with 28 kb of DNA independently expressing both full-length dystrophin and β-galactosidase. *Proc. Natl. Acad. Sci. USA* **93,** 5731–5736.
99. Siegl, G., Bates, R. C., Berns, K. I., et al. (1985) Characteristics and taxonomy of *Parvoviridae. Intervirology* **23,** 61–73.
100. Atchison, R. W., Casto, B. C., and Hammon, W. McD. (1965) Adenovirus-associated defective virus particles. *Science* **194,** 754–756.
101. Buller, R. M., Janik, J. E., Sebring, E. D., and Rose, J. A. (1981) Herpes simplex virus types 1 and 2 completely help adenovirus-associated virus replication. *J. Virol.* **40,** 241–247.
102. Kotin, R. (1994) Prospects for the use of adeno-associated virus as a vector for human gene therapy. *Hum. Gene Ther.* **5,** 793–801.
103. Flotte, T. R. and Carter, B. J. (1995) Adeno-associated virus vectors for gene therapy. *Gene Ther.* **2,** 357–362.
104. Flotte, T. R., Afione, S. A., Conrad, C., et al. (1993) Stable in vivo expression of the cystic fibrosis transmembrane conductance regulator with an adeno-associated virus vector. *Proc. Natl. Acad. Sci. USA* **90,** 10,613–10,617.
105. Kotin, R. M., Siniscalco, M., Samulski, R. J., et al. (1990) Site-specific integration by adeno-associated virus. *Proc. Natl. Acad. Sci. USA* **87,** 2211–2215.
106. Kotin, R. M., Menninger, J. C., Ward, D. C., and Berns, K. I. (1991) Mapping and direct visualisation of region-specific viral DNA integration site on chromosome 19q13-qter. *Genomics* **10,** 831–834.
107. Blacklow, N. R., Hoggan, M. D., Kapikian, A. Z., Austin, J. B., and Rowe, W. P. (1968) Epidemiology of adeno-associated virus infection in a nursery population. *Am. J. Epidemiol.* **88,** 368–378.

108. Blacklow, N. R., Hoggan, M. D., Sereno, M. S., et al. (1971) A seroepidemiologic study of adenovirus-associated virus infection in infants and children. *Am. J. Epidemiol.* **94,** 359–366.
109. Blacklow, N. R. (1988) Adeno-associated viruses of humans. In *Parvoviruses and Human Disease* (Pattison, J., ed.), CRC Press, Boca Raton, FL, pp. 165–174.
110. Chiorini, A., Wendtner, C. M., Urcelay, H., Saffer, B., Hallek, N., and Kotin, R. M. (1995) High efficiency transfer of the T cell co-stimulatory molecule B7-2 to lymphoid cells using high-titer recombinant adeno-associated virus vectors. *Hum. Gene Ther.* **6,** 1531–1541.
111. Walsh, C. E., Liu, J. M., Xiao, X., Young, N. S., Nienhuis, A. W., and Samulski, R. J. (1992) Regulated high level expression of a human γ-globin gene introduced into erythroid cells by an adeno-associated virus vector. *Proc. Natl. Acad. Sci. USA* **89,** 7257–7261.
112. Flotte, T. R., Conrad, C., Reynolds, T., et al. (1995) Preclinical evaluation of AAV vectors expressing the human CTFR cDNA [abstract]. *J. Cell Biochem.* **21A,** 364.
113. Robbins, P. D., Tahara, H., Mueller, G., et al. (1994) Retroviral vectors for use in human gene therapy for cancer, Gaucher disease, and arthritis. *Ann. NY Acad. Sci.* **716,** 72–88.
114. Riviere, I., Brose, K., and Mulligan, R. C. (1995) Effects of retroviral vector design on expression of human adenosine deaminase in murine bone marrow transplant recipients engrafted with genetically modified cells. *Proc. Natl. Acad. Sci. USA* **92,** 6733–6737.
115. Cornetta, K. (1992) Safety aspects of gene therapy. *Br. J. Haematol.* **80,** 421–426.
116. Wilson, C. A., Ng, T.-H., and Miller, A. E. (1997) Evaluation of recommendations for replication competent retrovirus testing associated with use of retroviral vectors. *Hum. Gene Ther.* **8,** 869–874.
117. Hacein-Bey-Abina, S., Le Deist, F., Carlier, F., et al. (2002) Sustained correction of X-linked severe combined immunodeficiency by ex vivo gene therapy. *N. Engl. J. Med.* **346,** 1185–1193.
118. Kim, V. N., Mitrophanous, K., Kingsman, S. M., and Kingsman, A. J. (1998) Minimal requirement for a lentivirus vector based on human immunodeficiency virus type 1. *J. Virol.* **72,** 811–816.
119. Miyoshi, H., Blomer, U., Takahashi, M., Gage, F. H., and Verma, I. M. (1998) Development of a self-inactivating lentivirus vector. *J. Virol.* **72,** 8150–8157.
120. Mitta, B., Rimann, M., Ehrengruber, M. U., et al. (2002) Advanced modular self-inactivating lentiviral expression vectors for multigene interventions in mammalian cells and in vivo transduction. *Nucleic Acids Res.* **30,** e113.
121. Koya, R. C., Kasahara, N., Pullarkat, V., Levine, A. M., and Stripecke, R. (2002) Transduction of acute myeloid leukemia cells with third generation self-inactivating lentiviral vectors expressing CD80 and GM-CSF: effects on proliferation, differentiation, and stimulation of allogeneic and autologous anti-leukemia immune responses. *Leukemia* **16,** 1645–1654.
122. Zufferey, R., Dull, T., Mandel, R. J., et al. (1998) Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery. *J. Virol.* **72,** 9873–9880.
123. Cheng, L., Chaidhawangul, S., Wong-Staal, F., et al. (2002) Human immunodeficiency virus type 2 (HIV-2) vector-mediated in vivo gene transfer into adult rabbit retina. *Curr. Eye Res.* **24,** 196–201.
124. Kobayashi, M., Iida, A., Ueda, Y., and Hasegawa, M. (2003) Pseudotyped lentivirus vectors derived from simian immunodeficiency virus SIVagm with envelope glycoproteins from paramyxovirus. *J. Virol.* **77,** 2607–2614.
125. Berkowitz, R., Ilves, H., Lin, W. Y., et al. (2001) Construction and molecular analysis of gene transfer systems derived from bovine immunodeficiency virus. *J. Virol.* **75,** 3371–3382.
126. Molina, R. P., Matukonis, M., Paszkiet, B., Zhang, J., Kaleko, M., and Luo, T. (2002) Mapping of the bovine immunodeficiency virus packaging signal and RRE and incorporation into a minimal gene transfer vector. *Virology* **304,** 10–23.
127. Lotery, A. J., Derksen, T. A., Russell, S. R., et al. (2002) Gene transfer to the nonhuman primate retina with recombinant feline immunodeficiency virus vectors. *Hum. Gene Ther.* **13,** 689–696.