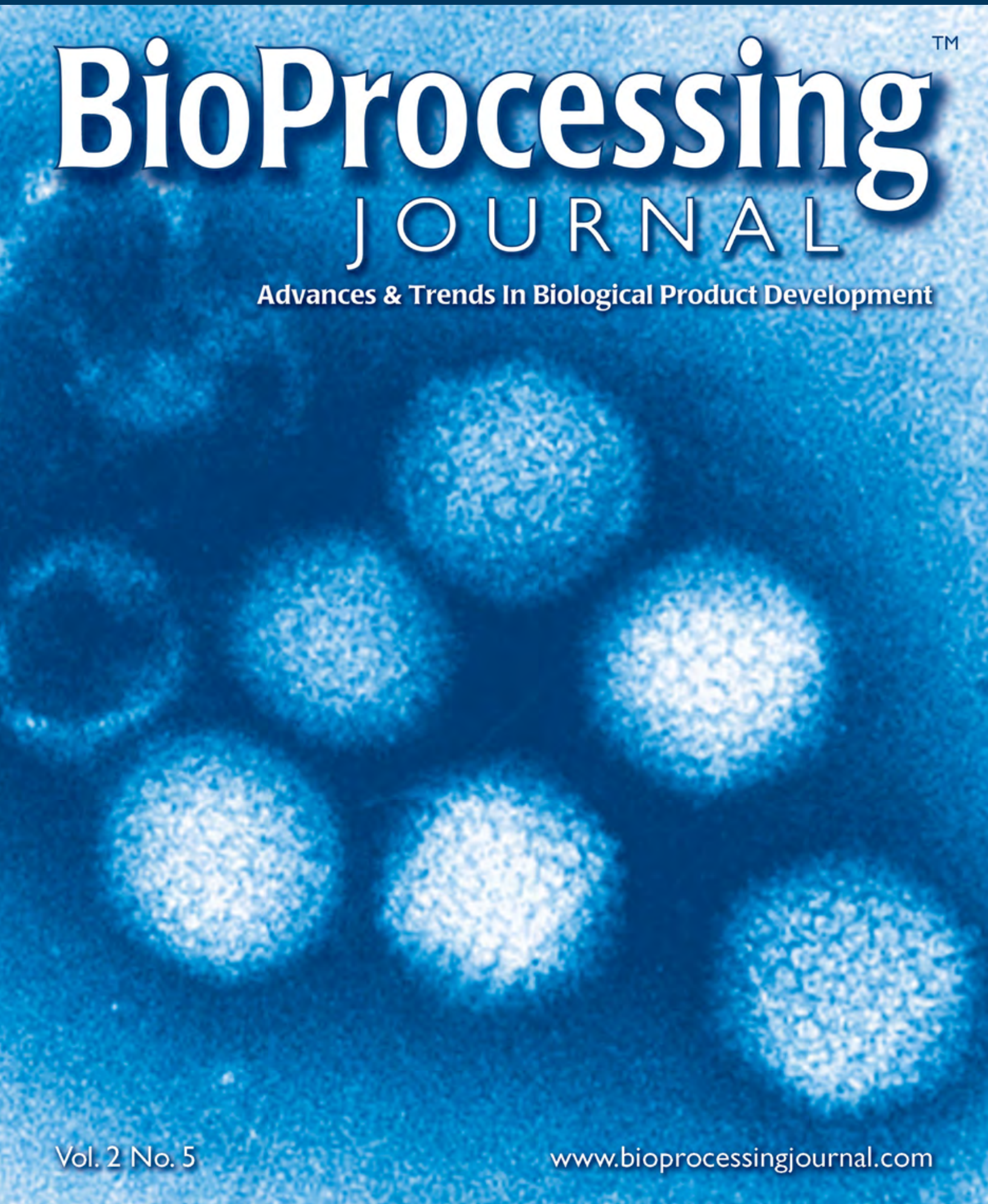
A publication of
The Williamsburg BioProcessing Foundation

September/October 2003

BioProcessing™ JOURNAL

Advances & Trends In Biological Product Development



Vol. 2 No. 5

www.bioprocessingjournal.com

Gene Therapy with Viral Vectors

BY DOUGLAS J. JOLLY

The modern era of interest in gene transfer as a methodology for treating disease began around 1985 with the first use and publication of mouse-based retroviruses that could transduce human cells.[1] In fact, the use of gene transfer as a clinically useful method is probably older than any other therapy commonly used today — it forms the basis for the vaccinia vaccination against smallpox, popularized in Western medicine by Jenner. Another antecedent is phage therapy for bacterial infection, which was largely but not completely superseded by antibiotics (although it may make a comeback in this era of drug-resistant pathogenic bacterial strains.)[2] Other examples include the other live viral vaccines: measles/mumps/rubella, polio, varicella, tuberculosis, influenza, the use of bacillus Calmette-Guérin (BCG) as a therapeutic for bladder cancer bone marrow transplants, and even the use of maggots to clean wounds.[3,4]

Why Gene Therapy?

The current picture of therapy or prophylaxis using gene transfer is closely related to the live vaccine idea. However, there are several concepts that gene therapy vectors can exploit that make new approaches to medicine possible. The most important of these are as follows.

Delivery of Large Biomolecules into Cells

First, gene therapy allows delivery of large biomolecules (such as proteins and nucleic acid) to the interior of cells, which is where most of the biochemical action is in animals and humans. It has been very difficult to get intact proteins into cells, and it is no coincidence that most recombinant biologics on the market are molecules that have a pharmacological effect when put into the circulation or are otherwise applied extracellularly. This includes many cytokines and hormones (e.g. IL-2, interferon alpha, growth hormone, and insulin) and also monoclonal antibodies, the latest addition to the biopharmaceutical arsenal. The experience and understanding of protein action has led to gene transfer as one way to deliver these proteins.

Specificity of Action

Second, and related to the intracellular action, is the specificity of action of biomolecules that typically have fewer unexpected side effects and toxicities than candidate small molecule drugs. This aspect is reinforced by the difficult, though not impossible, challenges in producing small molecule mimetics for macromolecules.[5]

Targeted Delivery

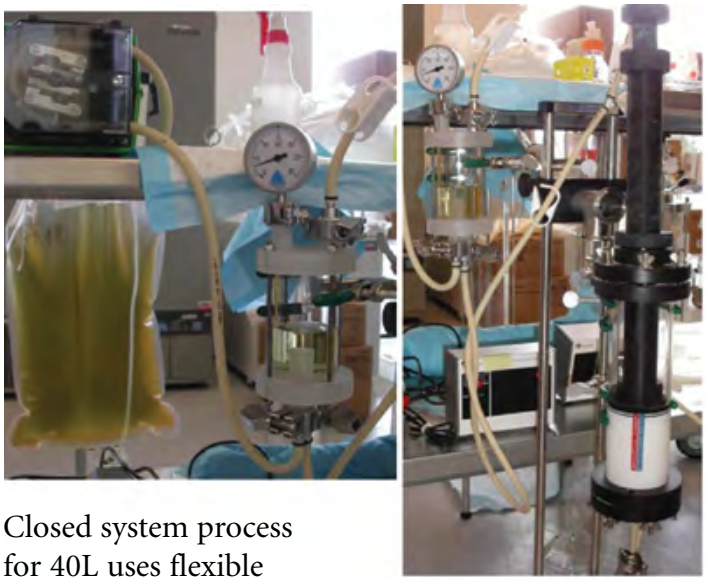
Third, there is an opportunity to avoid systemic exposure to protein or nucleic acid drugs by directing the genes, or the expression of genes, in various ways. Despite the above paean to specificity, many proteins are naturally pleiotropic, and can have different actions depending on local conditions. Ideally a protein, or any therapeutic agent, would only be present where it was necessary to elicit the therapeutic effect, as side effects of pharmaceutical agents always exist. The advantage of delivering a gene is that, unlike most small molecules and proteins, it stays put and delivers the biopharmaceutical payload

Douglas J. Jolly, Ph.D. *(d.jolly@biomedica-usa.com) is ceo at Biomedica Inc., San Diego, CA.*

A

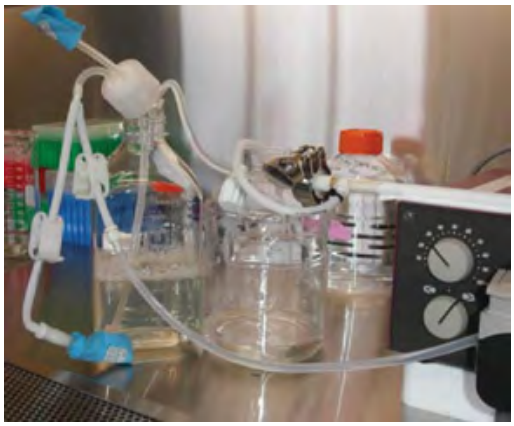
Scalable up to 20 Cell Factories

B



Closed system process
for 40L uses flexible
bag containers and sterile tubing welds
for clarification and ANX chromatography

C



Hollow fiber ultra-filtration

Figure 1: Typical equipment used for vector production at the 10–40 liter scale equivalent which yields about 10–40 human doses or 20–80 monkey doses of an EIAV lentiviral vector (30) designed to treat Parkinson's Disease.[29]

locally. At present, the most effective way to target a particular tissue is often by delivering the gene vector through a device such as a needle or catheter into the tissue. Other methods have had some success, at least in animal models. These include topical delivery, intravenous delivery to the liver and blood compartment, various forms of liquid delivery to lung tissue, introducing genes into cells *ex vivo* (e.g. T cells, $CD34^+$ cells) then returning these to the body, modifying surface proteins on vector particles to alter affinities and binding, and using tissue-specific promoters to limit expression to certain cell types.

High Potency

Finally, an underappreciated property of vectors based on modified viruses is that they are generally quite potent and, although they have had their share of manufacturing issues, the manufacturing scale per estimated human dose is moderate compared to many recombinant proteins (Fig. 1). For example, a typical dose of recombinant factor VIII (a crucial blood clotting protein) is 50–500 µg, whereas vectors are therapeutic at mass doses at least 100-fold lower than that, at least in animal models. Other proteins require higher doses in the milligram range; monoclonal antibodies are heading to even higher doses in the hundreds of milligrams. Gene therapy vectors, therefore, likely have a considerable mass advantage in the amount of material required for an effective human dose. As there are no gene therapy drugs approved yet (apart from the vaccines mentioned above), this advantage is necessarily not definitive — but the potential is definitely there. In fact, this accounts for the academic cottage industry in gene therapy, in which it is possible to manufacture the smaller quantities of material that constitute a human dose with academic dollars. This is not typically feasible for recombinant proteins.

Timing

As noted above, the modern concept of therapy by transfer of specific genes to specific tissues was first taken seri-

ously by the scientific and medical community around 1985, and the first clinical trial started in 1990.[6] The course of gene therapy, from high intellectual excitement at the new concepts to disappointment in the face of both foreseen and unforeseen difficulties, is reminiscent of the monoclonal antibody "magic bullets" story. Only in the last few years have monoclonal antibodies begun to receive regulatory approval and appear on the market. Most observers expect a similarly happy ending for gene therapy, so using the same timeline, gene therapy products should start to appear in the next five years. If true, this means these products should currently be in development and in the clinic. Indeed, there are a number of agents in clinical trials that are promising candidate products in cardiovascular disease, genetic disease, and even in that graveyard of new therapies, cancer. For example, an adenoviral vector fibroblast growth factor (FGF4) agent has shown Phase II efficacy data in angina pectoris, and it seems clear that hemophiliacs will be treated by AAV, retroviral, or other vectors encoding factors VIII or IX.[7-9]

In addition, the sole clear, published cure from gene therapy is the treatment of X-linked severe combined immunodeficiency syndrome (SCID).[10] In this study, bone marrow-derived hematopoietic stem cells from infants with the genetic disease were transduced with the gene for which they were deficient (the gamma chain of the IL-2 and IL-15 receptors). This syndrome leaves children without an immune system and hence very susceptible to infection. The treatment appears to be essentially a complete cure, allowing the children to lead normal lives. However, in a classic example of how risk and benefit often show up at the same time, two of nine children developed a leukemia that seems to be linked to the treatment by insertional activation of a gene associated with a translocation in cancer.[11] The affected children appear to be responding well to chemotherapy and are not dead, despite reports to the contrary. Although all instances of serious disease (especially in children) are tragic, it is clear that this therapy is a resounding success, albeit with a finite chance of serious side effects. If this therapy were not "Gene Therapy" it would likely be widely recognized as a success. The negative reaction from the public and regulatory authorities around the world is the flip side of the initial enthusiasm and heightening of expectations that seem inevitable with the appearance of any new clinical paradigm.

In cancer, progress with viral vectors has been on a broader front, and a number of agents have shown therapeutic effects at levels that suggest strong product potential. Many of these are based on cancer immunotherapy (which many believe will work), but no one is quite sure of which indications and with what agents exactly.[12] These include the expression of tumor-associated antigens, cytokines, and other immune stimulators such as CD40 ligands.[13-15] Cancer researchers are testing a number of different vectors, including vaccinia, adenoviruses, retroviruses, and DNA.

Another promising approach to cancer — currently in Phase III trials for head and neck cancer — is the use of adenoviral vectors expressing p53 to induce local tumor death.[16] Also, after a rocky start, some of the conditionally replicating oncolytic vectors (herpesvirus, for instance) have advanced into clinical trials.[17]

Finally, at least a few agents or approaches have such compelling animal data that it is easy to believe that these are products-in-waiting. These include the use of glial cell line-derived neurotrophic factor (GDNF) for Parkinson's disease, the adenoviral vector prime boost as an HIV vaccine strategy, and the use of lentiviral vectors encoding antiangiogenic factors as therapies for macular degeneration and diabetic retinopathy.[18-21]

So, What's the Catch?

A major consideration with gene therapy is that to effectively practice the technology, a cross-disciplinary team is needed to try to anticipate the multiple issues in different scientific disciplines that will inevitably occur. Although this is commonplace knowledge in drug development circles, it was often not the situation with the individuals and groups who pioneered the first gene therapy clinical trials. Some, though by no means all, of these types of expertise were mainly available in large drug companies. Expertise was (and remains) needed in virology, immunology, toxicology and pharmacology, the target diseases, process development and manufacturing of biologics, clinical trials, and the regulatory arena. However, not only were all these capabilities necessary, but they also had to be applied conceptually to a new technology (viral vectors) and the issues associated with drug development had to be worked out. Hindsight is 20/20, but it truly seems that some issues could have been better addressed. One example (but by no means the only one) is the issue of immune responses to viral vectors, particularly those to which most humans are already immune.[22] Although the consequences of this are still to be completely understood, the existence of such immune responses raises a number of questions such as:

- How often to readminister the agent?
- Do potencies suffer because of this phenomenon?
- What toxicities could be linked to administration in the face of a pre-existing immune response to the vector?

In general, immunologists were not heavily involved in the early days of gene therapy, despite the fact that quite a few approaches involved inducing immune responses to viral or tumor-associated antigens. It is certainly possible to find immunologists who say that, had it been up to them, they would never have started with such vectors. In fact, it turns out that excellent immunology is now being performed on the different vector systems, resulting in exciting possibilities for controlling whether or not immune responses to the vector or transgene arise, depending on which vector is used and how and where it is administered.[23] This is only one example, but it is fair to say that there are many cases in which the issues were underestimated or not fully appreciated. This is inevitable in pushing a new technology forward, but

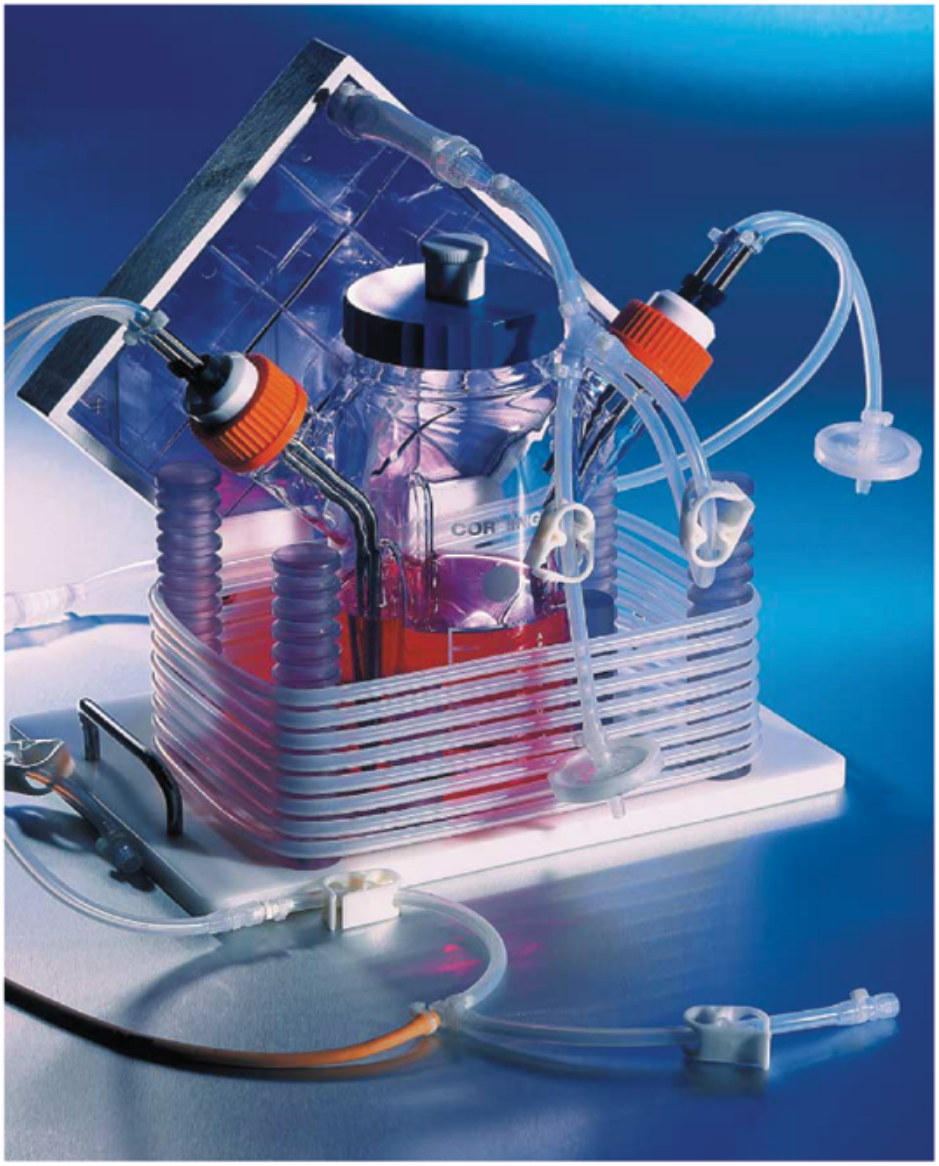
Table 1. Summary of properties of vectors that have been in the clinic

Vector	Strengths	Properties and limitations	Known toxicities and issues	Clinical areas of typical use	Manufacturing status	% current Gene Therapy trials to date (26)
1st generation adenoviral vectors	Easy to make, high levels of expression	Short term expression, limited doses	Relatively low limiting dose, pre-existing immune response, immunogenic	Cancer, limited duration expression	Straightforward, complementing cell lines for deficient viral functions (293, PER C6)	27%
Replication competent vectors- mainly adeno, but also herpes	Relatively easy to make, can be made cell type or tumor specific	The specificity still not that great	Potency seems lower than expected, replication can die out	Cancer	Straightforward	NA
AdenoAssociated Virus (AAV)	Long term expression, several serotypes may have improved properties	Seem quite well tolerated, tropism in vivo can be unexpected	Insertional mutagenesis issues have been raised no clinical evidence, pre-existing immunity appears not to be strong limitation	Genetic diseases, chronic diseases	Never been very easy, usually made by transient transfection on tissue culture cells	2.4%
Retroviral (MLV based)	Long term expression	Extensively used	Replication competent retrovirus contamination insertional activation of oncogenes in one trial, transduces only replicating cells	Infectious disease, cancer, genetic disease	Routine, continuous production from cell lines	34%
Lentiviral (HIV, FIV, BIV and EIAV based)	Long term expression, useful natural tropisms, transduces many non-replicating cells	Only one trial so far	Pre-existing immunity to HIV, mobilization by HIV, regeneration of replication competent lentivirus (hypothetical)	Infectious disease, chronic neurological disease	Transient transfection on 293 cells	0.15%
Herpes	Extensive under-standing of biology, BAC cloning has made manipulation of large genome much simpler	Neurotropic large genome in one format	Can be toxic unless very disabled; complex	Cancer, Pain	Packaging cell lines made, preparation still difficult	0.8%
Vaccinia/ Pox	Known entity- smallpox vaccine	Parent (non-recombinant) used on many people	Known to have serious side-effects at a low frequency, pre-existing immunity	Cancer, Infectious disease	Routine and robust	6.1%

Corning® E-Cube™ Culture System

An inexpensive, entry level bioreactor for anchorage-dependent cells

Presenting the E-Cube Culture System, Corning's cost efficient bioreactor system for growing large quantities of anchorage-dependent cells in parallel-plate CellCube® modules on the same treated polystyrene surfaces used in Corning flasks and roller bottles.



E-Cube Systems are ideal for:

- Scaling up cell production in a limited space with a simple, inexpensive, compact bioreactor with 8,500 cm^2 cell growth area in only a 25.4 cm x 35.6 cm footprint
- Learning bioreactor methodology, including making sterile connections, cell inoculating and harvesting, and monitoring metabolite utilization by cells
- Evaluating cell growth on Corning's parallel-plate CellCube modules before investing in the automated system
- Harvesting cells, not just culture medium. Unlike hollow fiber bioreactors, cells can both be harvested and viewed with a microscope in an E-Cube system.

The E-Cube System with the CellCube module is ideal for producing:

- Large amounts of cells or recombinant proteins
- Viral vaccines
- Viral vectors for gene therapy

E-Cube Systems are supplied with:

- An oxygenator
- A medium reservoir
- Multiple access ports
- All required tubing and fittings for connecting with CellCube modules (sold separately)
- You supply the peristaltic pump, CO_2 incubator, medium and cells.
- It is that simple!

For additional product information, please visit **www.corning.com/lifesciences**, or call 1.800.492.1110. Outside the United States, please call 978.635.2200.

Corning Incorporated
Life Sciences
www.corning.com/lifesciences

U.S.A.
t 800.492.1110
t 978.635.2200
f 978.635.2476

Europe
t 31 (0) 20-659-60-51
f 31 (0) 20-659-76-73

Japan
t 81 (0) 3-3586 1996/1997
f 81 (0) 3-3586 1291/1292

Latin America
t (52-81)-8158-8400
f (52-81)-8313-8589

CORNING
Discovering Beyond Imagination

it is possible that daylight could have dawned sooner. Fortunately, there is now a considerable cadre of individuals, mainly in biotechnology companies, who have the appropriate experience to get the balance right.

Another issue that has taken time to address is the relative complexities of these agents. It is well known that seemingly inconsequential changes to manufacturing processes can occasionally change the properties of biological drugs in unexpected ways. These were new agents, new processes, and the combination has been fragile. At the R&D stage, the differences between "good" preps (i.e. those that had the desired biological effect) and "bad" preps (those that did not) were often not apparent.

One take-home lesson from all of this seems to be that, in general, hoping for the best is a bad strategy. If there seems to be a possibility of a problem, it is best to assume that if you can think of it, it must be fairly obvious and therefore quite likely to happen. Some of these issues are listed below.

Scientific Difficulties with Viral Vectors that Might Have Been Anticipated

- Immunogenicity of foreign proteins, with intracellular delivery allowing class I responses
- Immunogenicity or preexisting immunity may limit readministration possibilities
- Other arms of the immune system (complement, cellular antiviral strategies)
- Tumorogenicity innate to extra DNA in nucleus
- Control of gene expression

Product Development Issues in Early Viral Vectors

- Defined reproducible material from novel manufacturing and testing procedures
- Difficulties in defining release tests that predict biological activity *in vivo*
- Effective design of clinical trials so that outcomes guide subsequent steps

A second lesson for gene therapy, and for developing new biopharmaceutical modalities in general, is the need for input as early as possible from individuals who are willing to work to understand the nitty-gritty details of the technology and its capabilities, then apply preexisting drug and biopharmaceutical development expertise.

Viral Vector Systems

This review describes some of the general issues associated with making and using viral vectors. However, different viral vector systems have different strengths and weaknesses. Good descriptions of the different viral (and nonviral) vectors have been published, and this is a broad subject for any detailed description, so no in-depth comparison of different vector systems is included here.[24,25] However, Table 1 describes the properties of the major vector systems that have been used in the clinic.

In addition to these vectors, many other viruses (including alphaviruses, SV40, measles, hepatitis B, polio, vesicular stomatitis virus, and baculovirus) have been proposed and used in the laboratory.[26] In general, any virus can be engineered to carry foreign genes. Every virologist has a scheme to make a vector out of his or her favorite virus, and usually there are one or more compelling reasons to become enthusiastic about a particular vector system. It is much more difficult to foresee what the issues will be as a novel product goes forward into drug development. For this reason it is easier to proceed with vectors for which experience exists, so that time and money are not spent creating that expertise. An extreme example is the use of first generation adenoviral vectors. These vectors were associated with the death in a clinical trial of a patient who received, in the hepatic artery, a dose of the vector (3.8×10^{13} particles) that is now recognized to be toxic by that route.[27] Subsequent data has indicated that this is likely a toxic dose by any intravascular route.[28] This is not the appropriate forum to comment on the ethical issues surrounding the incident, but one issue that became apparent was the need to standardize measurements of vector strength and potency, so that doses could be accurately described. The result is that a maximum tolerated dose of these vectors is well known: the 10^{13} to 10^{14} particle range for normal adults. For drug developers, this is welcome and useful knowledge.

Maximizing Success in Clinical Trials

Finally, for a gene therapy approach to a particular disease situation, what factors might facilitate success? The following is a partial list and may not cover every situation, but there is little doubt that these factors are important in determining the chances of a successful gene therapy product. Some represent hard-won experience and some are self-evident as risk reducers. For example, the first item refers to the idea that although systemic production of a recombinant protein has drawbacks as an approach, a large advantage is that this is where most known therapeutic proteins have actions. These items also imply the limitations that still exist for this powerful technology.

Considerations for a Successful Gene Therapy Approach

- The protein encoded by the vector is therapeutic when administered as protein (e.g. clotting factors)
- The therapy uses an accepted known mode of action (e.g. inhibits viral replication, ablates tumor cells, stimulates an immune response)
- The protein or other gene product can be made in the necessary amount
- The time frame for clinical action is appropriate for the specific vector
- The therapy is aimed at interfering with an accepted disease mechanism
- It is clear where to inject the vector
- The gene should be in an easily accessible tissue
- Precise control of gene expression is not necessary

It is usually not possible to have all of these, but the more of these conditions that can be satisfied, the more likely the drug/vector will be clinically successful.

Conclusion

Based on the timetable of other novel technologies such as monoclonal antibodies, the development of gene therapy products is on track and we can expect products to start reaching the market during the next five years. The current doubts that some have expressed concerning this diverse group of technologies called viral vectors are, in part, a reaction to initial overly optimistic projections of the time it would take to develop products. Gene therapy is alive and well and products are in the pipeline for a number of indications.

ACKNOWLEDGEMENTS

The author thanks Carlos Ibanez for the use of the pictures in Figure 1.

REFERENCES

1. Willis RC, Jolly DJ, Miller AD et al. Partial phenotypic correction of human Lesch-Nyhan (hypoxanthine-guanine phosphoribosyltransferase deficient) lymphoblasts with a transmissible retroviral vector. *J Biol Chem* 1984:259;7842-7849
2. Merril CR, Scholl D, Adhya SL. The prospect for bacteriophage therapy in Western medicine. *Nat Rev Drug Discov* 2003:2;489–497.
3. U.S. Food and Drug Administration (Center for Biologics Evaluation and Research). Vaccines licensed for immunization and distributed in the U.S. 2003. *www.fda.gov/cber/vaccine.licvacc.htm*
4. Chambers L, Woodrow S, Brown AP et al. Degradation of extracellular matrix components by defined proteinases from the greenbottle larva *Lucilia sericata* used for the clinical debridement of non-healing wounds. *Brit J Dermatol* 2003:148;14–23.
5. Air EL, Strowski MZ, Benoit SC et al. Small molecule insulin mimetics reduce food intake and body weight and prevent development of obesity. *Nat Med* 2002:8;179–183.
6. Anderson WF, Blaese RM, Culver K. The ADA human gene therapy clinical protocol: Points to Consider response with clinical protocol, July 6, 1990. *Hum Gene Ther* 1990:1;331–362.
7. Grines CL, Watkins MW, Helmer G et al. Angiogenic gene therapy (AGENT) trial in patients with stable angina pectoris. *Circulation* 2002:105;1291–1297.
8. Powell JS, Ragni MV, White GC II et al. Phase 1 trial of FVIII gene transfer for severe hemophilia A using a retroviral construct administered by peripheral intravenous infusion. *Blood* 2003:102;2038–2045.
9. Manno CS, Chew AJ, Hutchison S et al. AAV-mediated factor IX gene transfer to skeletal muscle in patients with severe hemophilia B. *Blood* 2003:101;2963–2972.
10. Hacein-Bey-Abina S, Le Deist F, Carlier F et al. Sustained correction of X-linked severe combined immunodeficiency by ex vivo gene therapy. *N Engl J Med* 2002:346;1185–1193.
11. Check E. Second cancer case halts gene therapy trials *Nature* 2003:421;305.
12. Ando D. Gene therapy and molecular immunology unite to give new life to cancer immunotherapy. *Mol Ther* 2002:5;665–667.
13. Mulryan K, Ryan MG, Myers KA et al. Attenuated recombinant vaccinia virus expressing oncofetal antigen (tumor-associated antigen) 5T4 induces active therapy of established tumors. *Mol Cancer Ther* 2002:1;1129–37.
14. Anon. Cell Genesys reports long-term survival data in Phase II trial of GVAX. *Expert Rev Anticancer Ther* 2002:2;245–6.
15. Battle TE, Wierda WG, Rassenti LZ et al. In vivo activation of signal transducer and activator of transcription 1 after CD154 gene therapy for chronic lymphocytic leukemia is associated with clinical and immunologic response. *Clin Cancer Res* 2003:9;2166–2172
16. Chada S, Mhashilkar A, Roth JA et al. Development of vaccines against self-antigens: The p53 paradigm. *Curr Opin Drug Discov Devel* 2003:6;169–173.
17. Nemunaitis J, Edelman J. Selectively replicating viral vectors. *Cancer Gene Ther* 2002:9;987–1000.
18. Kordower JH, Emborg ME, Bloch J et al. Neurodegeneration prevented by lentiviral vector delivery of GDNF in primate models of Parkinson's disease. *Science* 2000:290;767–773.
19. Gill SS, Patel NK, Hotton GR et al. Direct brain infusion of glial cell line-derived neurotrophic factor in Parkinson disease. *Nat Med* 2003:9;589–95.
20. Shiver JW, Fu TM, Chen L et al. Replication-incompetent adenoviral vaccine vector elicits effective anti-immunodeficiency-virus immunity. *Nature* 2002: 415;331–335.
21. Takahashi K, Saishin Y, Silva RL et al. Intraocular expression of endostatin reduces VEGF-induced retinal vascular permeability, neovascularization, and retinal detachment. *FASEB J* 2003:17; 896–898.
22. The Second Cabo Gene Therapy Working Group. Cabo II: Immunology and Gene Therapy. *Mol Ther* 2002:5;486–491.
23. Jooss K, Chirmule N. Immunity to adenovirus and adeno-associated viral vectors: Implications for gene therapy. *Gene Ther* 2003:10;955–963.
24. Friedman T, editor. *The development of human gene therapy*. New York: Cold Spring Harbor Laboratory; 1999.
25. Templeton NS, Lasic DD, editors *Gene Therapy — Therapeutic Mechanisms and Strategies*. New York: Marcel Dekker Inc; 2000.
26. Jolly DJ. Emerging viral vectors. In: Friedman T, editor. *The development of human gene therapy*. New York: Cold Spring Harbor Laboratory; 1999. p 209–240.
27. National Institutes of Health. Assessment of adenoviral vector safety and toxicity: Report of the National Institutes of Health Recombinant DNA Advisory Committee *Hum Gene Ther* 2002:13;3–13.
28. Reid T, Warren R, Kirin D. Intravascular adenoviral agents in cancer patients: Lessons from clinical trials. *Cancer Gene Ther* 2002:9;979–986.
29. Rohll JB, Mitrophanous KA, Martin-Rendon E et al. Design, production, safety, evaluation, and clinical applications of nonprimate lentiviral vectors. *Methods Enzymol* 2002;346:466–500.

Smart Solutions for Cellular Analysis

The Vi-CELL Series

- % Viability
- Total Cell Concentration
- Convenient Reagent Pack
- 21 CFR Part 11 Compliant

21 CFR Part II Compliant

The Vi-CELL family of instruments offers a suite of solutions for Cellular Analysis via unique real-time imaging capabilities.

Z Series

- Cell Enumeration
- Automated Calibration
- Dual Counting / Sizing Thresholds
- Population Dynamics
- Convenient Reagent Pack

Utilizing the world renowned 'Coulter Principle'. When accuracy COUNTS.

Visit us at our website: www.beckmancoulter.com

WORLD-CLASS GLOBAL SUPPORT: **AUSTRALIA** (61) 2 9844 6000 **CANADA** (1) 905 819 1234 **CHINA** (86) 106 515 6028 **EASTERN EUROPE, MIDDLE EAST, AFRICA** (41) 22 994 0707 **FRANCE** (33) 1 49 909083 **GERMANY** (49) 2151 333 625 **HONG KONG** (852) 2814 7431 **ITALY** (39) 2 25221 **JAPAN** (81) 3 5404 8359 **LATIN AMERICA** (1) (305) 380 3810 **MEXICO** (52) 5 559 1635 **NETHERLAND** (31) 2972 30630 **SINGAPORE** (65) 3393633 **SOUTH AFRICA** (27) 11 805 2014/5 **SPAIN** (34) 91 728 7900 **SWEDEN** (46) 8 564 85900 **SWITZERLAND** 0800 050 810 **TAIWAN** (886) 2 2378 3456 **U.K.** (44) 1494 429162 **U.S.A.** (1) 800 523 3713

BECKMAN COULTER™