Chapter 4.3

VECTOR-BASED ANTIVIRAL THERAPY

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Abstract: Viral and non-viral vector-based gene transfer and RNA-based inhibition are not only essential for basic molecular research in virology but can be developed into a valuable addition of traditional drug therapy. The efficiency of RNAi has largely supplanted brought other nucleic acid based drugs out of focus, although the central problem remains efficient and long-term delivery of these novel drugs. Expansion of virus-specific T-cells and (re)targeting of immune cells to virus infected cells are further promising ways to enhance antiviral defence. Vaccination strategies based on DNA as well as on vectors like recombinant modified vaccinia virus have reached early clinical stages.

1. INTRODUCTION

*It is only by Beelzebul, the prince of demons, that this man casts out demons.* Matth. 12:24

The feasibility of therapeutic gene transfer is widely accepted for cancer and genetic disease, and two thirds of human clinical trials were as yet performed in cancer patients. Herpesviruses, lentiviruses, papillomaviruses and hepatitis B and C viruses (HBV, HCV) all cause significant burden of disease in man. They establish persistent infections that are in many aspects similar to genetic disease or cancer, and several viruses are in fact carcinogenic. Similar attempts as in cancer gene therapy have thus been applied to viral infections, while the success of vaccination against infectious disease has on the other hand prompted studies to vaccinate against cancer. The majority of studies in humans that use gene transfer in infectious disease (about
7% of more than 1000 trials world wide) are HIV-related (68 of 72 regis-
tered trials on infectious disease, as of June 2005); the few others are on im-
munotherapy directed against Cytomegalovirus (CMV), Epstein Barr virus 
(EBV) and HCV (The Journal of Gene Medicine Clinical Trial Site, 2005; 
Office of Biotechnological Activities at NIH and the Recombinant DNA 
Advisory Committee, 2005). The ingenuity of researchers has come up with 
an abundance of antiviral targeting strategies, including promising recent 
developments such as RNA interference, strategies to specifically direct an 
antiviral immune response toward infected cells, and vaccination procedures 
that involve gene-modified antigen presenting cells. Many techniques are 
still in a preclinical or conceptual stage. The major hurdle of most vector-
based approaches remains the delivery of the therapeutic principle to the 
(virus-infected) target cell. Since preemptive application of antiviral gene 
transfer will more than likely remain an absolute exception in the foreseeable 
future, the viral foe will usually have a head start; in addition, for reasons of 
biosafety, the carefully controlled therapeutic vector has a fundamental dis-
advantage versus the spread of the infectious agent which is not hindered by 
such considerations.

2. VECTORS FOR ANTIVIRAL THERAPY

“It’ll be great to have polymers if they work, but I don’t think they’re 
efficient enough yet — viruses have learned to do this 
over millions of years” (Inder Verma, in: Clayton, 2004)

This review will focus on potential treatments that involve transfer of a 
plasmid or viral vector based expression cassette as the therapeutic principle. 
Some of the approaches discussed here are also being developed for direct 
short term treatment, such as synthetic nucleic acids and proteins.

The final goal of efficient and selective transduction of target cells with a 
therapeutic vector in the patient in vivo is not in sight. Current transfer sytms 
lack sufficient target cell selectivity, although various attempts are being 
made to adapt existing vector systems (Tab. 1). These include pseudotyping 
retro- or lentiviral particles, modifying retroviral envelope proteins (Sandrin 
et al., 2003; Verhoeyen and Cosset, 2004), construction of tropism-modified 
adeno-viruses (Mizuguchi and Hayakawa, 2004) or Adeno-associated virus 
(AAV) vectors (Buning et al., 2003), or integration of modified glycopro-
teins into herpesviruses (reviewed in Grandi et al., 2004).
2.1 Integrating viral vectors

"With AAV and retrovirus vectors you’re just rolling the dice"
(William M. Pardridge, in: Clayton, 2004)

The successful correction of X-linked severe combined immunodeficiency (SCID-X1) in young children by retroviral transfer of the cytokine receptor common gamma chain (γc) (Hacein-Bey-Abina et al., 2002) had been greeted enthusiastically as the fulfillment of the promises gene therapy had not been able to keep for more than a decade. It was soon followed by a tragic setback when three children developed treatment-associated T-cell leukemia that was associated with LMO2 gene expression, very likely the consequence of insertional activation by the retroviral vector (Hacein-Bey-Abina et al., 2003; Kaiser, 2005).

Insertion mutagenesis is the most significant problem of the otherwise convenient and widely used integrating vectors (Baum et al., 2003). Retroviral insertion mutagenesis had been observed previously in animal models; rhesus monkey infected by a retroviral vector contaminated with replication competent retrovirus developed T-cell leukemia (Donahue et al., 1992), and retroviral mutagenesis was later also recognized in murine models that allowed for longer observation times (Li et al., 2002b). It was recently reported for the first time for a non-primate equine infectious anemia virus (EIAV) based lentiviral vector (Themis et al., 2005). Why have those side effects not been observed before? It has been estimated that in the French trial the number of retroviral insertion events exceeded those of all previous studies together. High vector dose, usage of a conventional vector with complete LTR, and the growth stimulatory properties of the transduced γc gene, plus increased viability and engraftment of gene-modified cells may in summary have precipitated the occurrence of side effects. Compared to the prognosis of SCID-X1 patients in which matched related donors are unavailable, however, the current survival rates and outcome in the remaining patients of the French and related gene therapy trials represent a significant advance in the treatment of this severe disease (Baum et al., 2004).

Dose-finding will be important, as is development/application of newer generations of retro- and lentiviral vectors that will improve the outcome. These are self-inactivating (SIN) due to deletion of the enhancer elements in the LTR U3 region and can therefore avoid the direct activation of genes in the vicinity of the integration site. Nevertheless, dose-dependent genotoxicity by insertional gene inactivation is likely associated with any integrating viral vector. Safety concern might be overcome by including suicide genes that allow ablation of vector-transduced cells.
### Table 1. Viral Vectors

| Advantage                                      | Disadvantage                                                                 | Possible solution                                                                 |
|------------------------------------------------|------------------------------------------------------------------------------|----------------------------------------------------------------------------------|
| **Adenovirus**                                 |                                                                              |                                                                                  |
| High capacity                                  | Large and complex genome                                                     | Recombination with Ad5 sequences from HEK293 cell line                           |
| High Titers                                    | Recombination with Ad5 sequences from HEK293 cell line                       |  
| Transduction of resting, differentiated as well as proliferating cells | No active replication of vector genome: dilution effect in proliferating cells | Packaging cell lines with minimal sequence overlap                                |
| No integration into host cell genome           | No long-term persistence: repeated applications necessary                    |                                                                                  |
| Direct in vivo application possible             | Toxicity at high doses (Gelsinger case)                                      | Establish secure dose                                                            |
|                                                |                                                                              | Inflammation after repeated application                                          |
|                                                |                                                                              | Sequential use of alternative vector serotypes                                   |
|                                                |                                                                              | Preexistent immunity reduces efficiency:                                         |
|                                                |                                                                              | High seroprevalence of adenovirus infection, multiple serotypes                  |
|                                                |                                                                              | Vectors based on unrelated animal adenoviruses                                  |
|                                                |                                                                              | Vectors based on unrelated animal adenoviruses                                  |
|                                                |                                                                              | Tissue specific and/or polarized CAR expression                                  |
|                                                |                                                                              | Establish secure dose                                                            |
|                                                |                                                                              | Inflammation after repeated application                                          |
|                                                |                                                                              | Sequential use of alternative vector serotypes                                   |
|                                                |                                                                              | Preexistent immunity reduces efficiency:                                         |
|                                                |                                                                              | High seroprevalence of adenovirus infection, multiple serotypes                  |
|                                                |                                                                              | Vectors based on unrelated animal adenoviruses                                  |
|                                                |                                                                              | Vectors based on unrelated animal adenoviruses                                  |
|                                                |                                                                              | Tissue specific and/or polarized CAR expression                                  |
|                                                |                                                                              | Establish secure dose                                                            |
| Establish clinical vector                      | Established side effects                                                     |                                                                                  |
| **Retrovirus**                                 |                                                                              |                                                                                  |
| Small genome, simple structure:                | Recombination with complementing sequences in packaging cell lines: high risk of insertion mutagenesis by replication competent retroviruses (RCR): |                                                                                  |
| Easy construction of complementing packaging lines | Improved packaging cell lines with separate transgenes                       |                                                                                  |
|                                                | Reduced homologies by codon wobbling                                          |                                                                                  |
|                                                | Control for RCR                                                              |                                                                                  |
| In vivo gene transfer possible                 | Murine retrovirus vectors inactivated by primate complement                  |                                                                                  |
| No mobilization by primate Lentiviruses or human endogenous retroviruses (low sequence homology) | Reduced homologies by codon wobbling                                          |                                                                                  |
| Titers between $10^7$ and $10^7$ cfu/ml (concentration possible, intermediate stability of viral particles) | Control for RCR                                                              |                                                                                  |
| Colinear integration (LTR-gene-LTR):           | Transgene copy number correlates with expression (and risk for insertion mutagenesis) |                                                                                  |
| intact structure of integrated expression cassette |                                                                              |                                                                                  |
| Transduction depends on proliferation:        | Transduction depends on proliferation:                                     |                                                                                  |
| Targeted transduction of proliferating (tumor) cells | T-cell stimulation to proliferation increases HIV replication                 |                                                                                  |
| Stable integration into host cell genome:     | No integration in resting/terminally differentiated cells                    |                                                                                  |
| Transgene is preserved in further cell divisions | Introduce lentiviral elements for active nuclear transport of preintegration complex |                                                                                  |
| Establish clinical vector                      | Insertion mutagenesis: Oncogene activation, Tumor suppressor inactivation     |                                                                                  |
|                                                | Selection against harmful integrants (?)                                     |                                                                                  |
|                                                | Targeted integration                                                         |                                                                                  |
|                                                | Self-inactivating vectors                                                    |                                                                                  |
| Establish clinical vector                      | Established side effects                                                     |                                                                                  |
## Table 1. continued

### Lentivirus
- As above but
- Infection of non-dividing cells
- Possibly lower risk of insertion mutagenesis?
- Mobilization by superinfecting lentiviruses
- Parental viruses are primate pathogens

### Herpesvirus
- High packaging capacity
- Minimal vectors with reduced set of viral genes
  - Amplicons
- Active episomal persistence
  - Many unknown functions
  - Unknown pathogenicity factors
  - Tumorigenic potential of parental virus (EBV, HVS)
  - Further attenuation
  - Further research
- Broad tropism in primary infection
- Specific tropism of persistent infection
  - Neurotropic (HSV-1)
  - B-cells (EBV)
  - T-cells (H. saimiri)
- Transduction of resting, differentiated as well as proliferating cells
  - Recombination with persisting wildtype Herpesviruses
  - Vectors based on unrelated animal herpesviruses
- First clinical trials with oncolytic HSV-1 vectors
  - Preexisting Immunity: High seroprevalence of human infection
  - Other than HSV-1: Mostly experimental

### Adeno-associated Virus
- Small genome, simple structure
- Stable virus particles
  - Helper virus required (Adenovirus, HSV)
  - Pathogenicity/Immunogenicity of helper virus
  - Define helper functions: separate expression of helper function in producer cells
- High Titers possible (10^{12}/ml)
  - Concentration and purification from helper virus by ultracentrifugation necessary
  - Packaging lines with conditional Rep-expression
- Transduction of resting, differentiated as well as proliferating cells
  - Rep-overexpression toxic: only transient packaging systems.
  - Insufficient knowledge of Rep-function
- Stable Integration into target cell genome
  - Insertion mutagenesis
  - Stable integration in Chromosome 19 is infrequent:
    - requires high MOI and Rep-protein
  - Lack of integration in absence of Rep: Episomal persistence for limited time period, Dilution effect in proliferating cells
- Apathogenic properties of parental AAV and Dependoviruses
  - Preexisting Immunity:
  - High seroprevalence of natural parvovirus infection
However, expression of prodrug-activating genes can cause undesired elimination of transduced cells when the suicide gene is recognized by the immune system (Riddell et al., 1996). Furthermore, in the context of a transformation and replication competent rhadinoviral vector, the Herpes simplex virus thymidine kinase (HSV-TK) gene unexpectedly enhanced viral pathogenicity in primates (Hiller et al., 2000a), despite having been able to eliminate transduced cells in vitro (Hiller et al., 2000b).

Dependoviruses and Adeno-associated virus (AAV) vectors have not been commonly associated with side effects from integration (Bell et al., 2005), although tumors have been observed after AAV-transduction in mice (Donsante et al., 2001). Site specific integration of AAV-vectors at chromosome 19q13, which was previously considered the theoretical basis for long-term AAV gene transfer, is now recognized to be a rather infrequent event. Although it occurs at the high multiplicities of infection in nature, it requires approximately 100 to 1000 infectious particles per target cell after transduction with AAV vectors (Duan et al., 1998; Schnepp et al., 2003; Nakai et al., 2001). Reports by the Kay group provided insights into the nature of rAAV2 vector integration into chromosomes in quiescent somatic cells in animals and human subjects (Nakai et al., 2003), describing the host chromosomal effects of rAAV2 integration in mice. This result was greeted with mixed enthusiasm (Kohn and Gänbacher, 2003), but complemented earlier data from tissue culture (Miller et al., 2002). An extended analysis of AAV-integration sites in mice confirmed that AAV has the potential to act as an insertional mutagen (Nakai et al., 2005; Miller et al., 2005), although it does not seem to induce chromosome breaks by itself (Miller et al., 2004).

2.2 Episomal vector development

Episomal persistence and replication in the infected cell is a shared characteristic of the Herpesvirus and Polyomavirus life cycle, though integration occurs and is associated with significant pathology in humans (cervical/genital/skin cancer by human papillomaviruses) or chicken (Marek’s Disease Herpesvirus, MDV). EBV integration has been occasionally described in cultured cell lines but is considered a rare event in vivo (Gulley et al., 1992; Delecluse et al., 1993). Herpesviruses other than MDV do not regularly integrate and possess a number of attractive features such as large packaging capacity, a mechanism for active replication and segregation of the viral episome, a broad host range, and infection of non-dividing cells. This encouraged the development of a number of episomally persisting vectors based on Herpes simplex virus (HSV), CMV, EBV and the animal Herpesvirus saimiri (Cotter and Robertson, 1999; Mazda, 2002; Conese et al., 2004; Oehmig et al., 2004; Doody et al., 2005; Wieser et al., 2005).
Vectors build on RNA-virus replicons such as Alphavirus lack the capability for long-term persistence. Since they elicit a strong immune response, they will possibly find an application in immunotherapy and vaccination studies, where only transient expression is required and their immune stimulating properties may be advantageous.

2.3 Nonviral vectors

Recombinant or DNA-based vaccines hitherto have not made their way into advanced clinical testing, nor are recombinant viral vector-based vaccines licensed in Europe or the US. Nevertheless, transfer of naked DNA into skin or muscle has been successfully applied for DNA-vaccination studies in animals, also in combination with booster strategies that involve viral expression vectors (Yang et al., 2003); this has been able to provide protection from Filovirus challenge, and similar to adenoviral vector expressed Ebola virus glycoprotein alone rapidly protects primates (Sullivan et al., 2003).

Encapsulated forms of DNA have been delivered successfully in vivo to the liver or joints; transfer using cationic or receptor targeted liposomes is nonetheless inefficient, as the vehicle is frequently cleared from the circulation by phagocytic cells before the target is reached. Ex vivo transfer of DNA by lipids or electroporation, also in a flow-through format, may be possible and a practicable way of nonviral gene transfer (summarized in Rössig and Brenner, 2004).

3. VIRUS-SPECIFIC DNA- OR RNA-TARGETING STRATEGIES

There are basically two lines of attack, (A) directed against the exogenous target such as RNA-virus genomes and viral transcripts and, (B) to interfere with transcripts of host cell cofactors that are important for the invasion, replication, or pathogenicity.

DNA or RNA-targeting drugs have the advantage that by their sequence-specific action they are much easier to design compared to drugs that need to interact with structured targets such as cellular or viral proteins (Tab. 4.3.2). Multiple sequence targets can be combined in one formulation, although efficiency might be decreased by saturation, e.g. of RNA-interference (RNAi) pathways. The initial assumption that the non-proteinaceous nature of nucleic acid-based drugs prevents host immune response that would otherwise limits efficacy, did not hold true. Innate immunity is also activated
via Toll-like receptors (TLR) that recognize CpG motifs and dsRNA, resulting in stimulation of the interferon system.

3.1 Delivery of nucleic acids

The most important barrier for all nucleic acid-based treatments is their intrinsic low capacity to cross the cellular membrane, as they must be delivered into the inside of the cell. Before they can arrive there in sufficient amounts, they must remain stable and should have no toxicity in the host. Ribozymes have not been approved for clinical use so far, and the only licensed antisense DNA drug (Fomivirsen, which is directed against human CMV for treatment of retinitis) is not a great commercial success due to more reliable alternative treatments. After delivery across the cell membrane, the drug has to be released e.g. from the endosome and find its way to its target, which has to be structurally accessible. Compared to short-lived transfer of synthetic molecules, it seems therefore more attractive to perform vector-mediated transfer of expression cassettes for enzymatically active ribozymes, decoy molecules and specifically for siRNA-precursors (below), which also use physiological cellular mechanisms for transport and processing.

3.2 miRNA

Cellular micro-RNAs (miRNAs) are an ancient physiological mechanism for posttranscriptional gene silencing (PTGS). PTGS by miRNA or RNA-interference (RNAi) is a common eukaryotic defense pathway conserved all the way from plants over fungi to animals. It was first noted more than a decade ago in plants, then fungi, nematodes, flies and higher animals. The underlying molecular mechanism of miRNA and RNAi are highly similar and have been elucidated mostly by the work of Tuschl and colleagues (Tuschl et al., 1999; Zamore et al., 2000; Elbashir et al., 2001a, 2001b).

Briefly, RNA polymerase II transcribes mammalian miRNA genes into long primary miRNAs (pri-miRNAs), which are then processed in the nucleus into 70–80-nucleotide hairpin-like precursor miRNAs (pre-miRNAs) by Drosha, a nuclear RNAse III which is in complex with DGCR8, an RNA binding protein. Exportin-5 together with its cofactor Ran-GTP transport pre-miRNAs to the cytoplasm, where they are processed by the RNAse III Dicer into mature 22-nucleotide duplex miRNAs. The Dicer containing complex processes long double-stranded RNA precursors into the small interfering RNAs (siRNAs) that mediate RNAi. Beginning at the duplex end with the lower thermodynamic stability, miRNA duplexes are unwound, and the miRNA strand that has its 5’ terminus at this end is the future mature miRNA (also called guide RNA). Unwound mature miRNAs are
then incorporated into a miRNA ribonucleoprotein complex analogous to the RNA-induced silencing complex (RISC). The miRNA/RISC then downregulate the target by translational inhibition or target mRNA cleavage. This is determined by the degree of complementarity between the miRNA and target gene, in combination with Argonaute proteins, which are present in RISC. All Argonaute proteins can bind miRNAs and siRNAs, and Argonaute2 (eIF2C2) is the catalytic component of the RISC that mediates targeted RNA cleavage. Near-perfect complementarity results in cleavage, followed by general RNA degradation of the targets, while partial complementarity causes translational inhibition, though the exact mechanism for translational inhibition is not known. Translational inhibition seems more common than miRNA-directed cleavage in animals (reviewed in Wienholds and Plasterk, 2005; see also Chapter 1.3, Figure 5). Given that the recognition of miRNA usually involves 1-2 mismatches to its target sequence, current bioinformatic prediction methods for miRNA and their potential targets are not dependable, and detection of miRNAs, their targets, and validation of target specificity so far mostly rely on experimentation.

As indicated, PTGS by RNAi is a conserved mechanism of antiviral defense, all the way from plants to animals. This is indicated by the finding that several viruses have evolved proteins interfering with RNAi. Viral suppressors of PTGS were first recognized in plants (reviewed in Voinnet et al., 1999; Roth et al., 2004), and plants in response seem to have evolved secondary defense mechanisms targeting these viral proteins (Li et al., 1999; Savenkov and Valkonen, 2002). Likewise, suppression of RNA interference by animal viral pathogens was recently described (Li et al., 2002a; Lichner et al., 2003; Bucher et al., 2004; Delgadillo et al., 2004; Li et al., 2004; Soldan et al., 2005).

Interestingly, it was recently found that some viruses, mostly large-genome DNA-viruses, have evolved or acquired expression of miRNAs (Pfeffer et al., 2004, 2005; Samols et al., 2005; Cai et al., 2005). A first viral miRNA function has been revealed in that the simian polyomavirus 40 encoded miRNA regulates large T antigen expression and reduces susceptibility of infected cells against cytolysis by cytotoxic T-cells (Sullivan et al., 2005). The cellular or viral targets of other virus encoded miRNAs are unknown.
3.3 RNA interference

“RNAi is what antisense never was. It’s robust and reproducible, and it exists in nature” (Inder Verma, in: Clayton, 2004)

Within a few years, the technique of RNAi has been widely and successfully employed in the dissection of cellular and viral functions, and the enormous potential for therapeutic application has been rapidly recognized (reviewed in Shankar et al., 2005; Stevenson, 2004). Briefly, the concept has been successfully applied to therapeutic approaches directed against HIV (Berkhout, 2004; Boden et al., 2004; Cullen, 2005), HCV (reviewed in Chapter 1.3 by Frese and Bartenschlager), SARS-CoV (Wu et al., 2005), and using RNAi was even suggested as a way to breed non-permissive mosquitoes in order to interrupt Dengue virus transmission (Sanchez-Vargas et al., 2004).

Table 2. Nuclei-Acid based Strategies

| Strategy        | Advantages                                                                 | Disadvantages                                                                 |
|-----------------|----------------------------------------------------------------------------|-------------------------------------------------------------------------------|
| Antisense Oligonucleotides | Straightforward design and production, inexpensive | Protein binding possible (Aptamer activity)                                   |
|                 | Can be modified for systemic delivery                                      | Exogenous delivery of synthetic molecules necessary                         |
|                 | Introns and exons as possible targets                                      | Off-target effects possible                                                  |
| Ribozymes       | Discrimination of single nucleic acid polymorphisms (SNP)                  | Limited target choice, GUC triplet required                                   |
|                 | Introns and exons, subcellular compartments as targets                    | Protein binding possible (Aptamer activity)                                  |
|                 | \textit{In vitro} and \textit{in vivo} production possible                | Dependent on \textit{in vivo} folding and compartment                        |
|                 | Target specificity can be changed                                           |                                                                              |
|                 | Simple catalytic domain                                                    |                                                                              |
|                 | Can correct defects                                                       |                                                                              |
| DNAzymes        | Straightforward design and production, inexpensive                        | Protein binding possible (Aptamer activity)                                  |
|                 | Catalytic properties                                                       | Dependent on \textit{in vivo} folding and compartment                        |
|                 | Can be modified for systemic delivery                                      | Exogenous delivery of synthetic molecules necessary                         |
|                 | Introns and exons as possible targets                                      | Off-target effects possible                                                  |
| RNA-interference | Based on natural mechanism                                               | Only mRNA as target                                                         |
|                 | Straightforward design and production, relatively inexpensive             | Induction of Interferon possible                                            |
|                 | Effective at low concentration                                             |                                                                              |
|                 | Exogenous and endogenous delivery possible                                 |                                                                              |
|                 | Tissue specific expression possible                                        |                                                                              |
|                 | Sequence specific: some discrimination of nucleic acid polymorphisms (SNP)|                                                                              |

Modified from (Scherer and Rossi, 2003)
In most cases RNA interference is efficient and highly specific. However, induction of the interferon system by the Toll-like receptor (TLR) pathway can result from recognition of byproducts of siRNA production or the specific siRNA itself. On the other hand, TLR pathway activation by a given siRNA can be experimentally ruled out in vitro and in vivo in various models, and has not been shown to interfere with the degree or specificity of RNA interference. More difficult to overcome, but possibly less severe, is the interference with off-target cellular RNAs that has been observed sporadically (Jackson et al., 2003; Scacheri et al., 2004). The potential off-target effects of the respective therapeutic RNA must therefore be studied extensively in the homologous species, e.g. by genome-wide expression analysis. A further aspect is the evaluation of such strategies in vivo: the high sequence specificity of RNA interference will limit the predictive value of any preclinical animal model, since it is not unlikely that a given siRNA will find different off-targets in other host genetic backgrounds.

The delivery of the therapeutic principle to the target cell faces similar problems as with other aforementioned techniques that rely on transfer of short nucleic acids or vector-mediated transfer. Synthetic precursor RNAs need to be protected from nucleases and can be delivered using techniques such as liposomes or ligand mediated delivery across the membrane of the target cell. Some cell types can also take up nucleic acids directly. Nevertheless, the effects of synthetic RNA will be transient and short-lived. Sustained as well as regulated intracellular expression of short-hairpin structured siRNA precursors (shRNA) has been achieved by a variety of integrating and non-integrating viral vectors, along with their particular advantages and disadvantages (Tab. 1). Widely used examples are retro- and lentiviral SIN vectors where the shRNA expression cassette is located in the 3’LTR region; two copies of the shRNA cassette are integrated into the target cell after reverse transcription has duplicated the 3’LTR (Barton and Medzhitov, 2002; An et al., 2003; Wiznerowicz and Trono, 2003; Lee et al., 2003); AAV have also been used (Tomar et al., 2003).

### 4.3. Vector-based Antiviral Therapy

The targeting of infected cells by cytotoxic vectors is conceivable via viral or cellular proteins that are presented exclusively or preferentially at the cell surface. Productive infection with enveloped viruses is usually associated with expression of viral (glyco-)proteins at the surface of cells. Infected cells frequently also show upregulated expression of specific surface molecules such as the MHC-I-like ligands for NK-cell recognition via NKG2D.
Unsurprisingly, viruses have evolved evasion mechanisms (Alcami and Koszinowski, 2000a, 2000b, 2000c; Cerwenka and Lanier, 2003; Lodoen et al., 2004; Krmptotic et al., 2005). Glycoprotein-deficient rhabdoviruses modified to express CD4, or CD4 plus CXCR4 have been designed as “Magic bullets” for selective infection (Mebatsion et al., 1997; Mebatsion and Conzelmann, 1996) or even killing (Schnell et al., 1997) of cells displaying HIV envelope glycoprotein at their surface. Analogous targeting could be achieved by retroviruses pseudotyped with CD4 or CXCR4 (Endres et al., 1997; Somia et al., 2000), or anti-CCR5-scFv pseudotyped Sindbis vectors (Aires et al., 2005). However, neither strategy has been translated to clinical applications so far.

5. EXPRESSION OF ANTIVIRAL MOLECULES

5.1 Dominant negative proteins or receptors

Soluble forms of the main HIV-receptor CD4 can block infection and moreover CD4-TCRζ fusion proteins have been suggested, in which viral binding to the chimeric molecule will conditionally induce cytotoxicity before the virus can establish infection and replicate. Both approaches suffer from the fact that primary isolates are less dependent on CD4 and can infect via alternative receptors. An intracellular variant of CD4 (CD4-KDEL) that is retained in the endoplasmatic reticulum can block viral glycoprotein-transport (Buonocore and Rose, 1993). Intrakines are modified chemokines such as MIP-1α or RANTES designed to be retained in the ER; this results in ER-retention of the HIV coreceptor CCR5, reducing its expression at the cell surface.

Mutated forms of viral proteins that can exert trans-dominant negative effects on viral replication have been described for HIV Tat and Rev, and also for Gag, Env, and the protease. Clinical trials with transdominant-negative RevM10 showed increased survival of RevM10-expressing T-cells in AIDS-patients. A strong immune response to the Rev protein was observed, initiating further investigations into Rev-based therapeutic vaccines (Bevec et al., 1992, 1996). Cellular transport factors such as eIF-5A are required for Rev-mediated nuclear export. Dominant-negative mutants of eIF-5A have been shown to inhibit the replication of HIV, and eIF-5A can also be a target for small molecule inhibitors of hypusine modification (Hauber et al., 2005).
5.2 Intracellular expression of Antibodies or Intrabodies

Antibodies or single chain antibodies derived from the variable regions of the immunoglobulin heavy and light chains (intrabodies) have been derived against viral targets such as Env, Tat, Rev, reverse transcriptase, integrase or cellular targets required for efficient HIV-replication. Intrabodies targeting the viral reverse transcriptase and integrase can counteract early in the HIV lifecycle and theoretically can prevent HIV integration and as a result confer immunity from infection. Humanization of the primarily murine monoclonal antibodies will be necessary to decrease the immunogenic potential. Intracellular expression of LANA-specific intrabodies inhibited persistence of Kaposi sarcoma associated herpesvirus (KSHV, HHV8) in lymphoma cells (Corte-Real et al., 2005), indicating that such an approach could eventually be used to overcome persistent herpesvirus infections.

5.3 Virus-specific expression of prodrug-activating enzymes

Gene-directed enzyme prodrug therapy or gene-prodrug activation therapy relates to the transfer and expression of enzymes that metabolize nontoxic prodrugs and convert them into active cytotoxic substances. HSV thymidine kinase (HSV TK), Cytochrome P450, or Cytosinedeaminase from E. coli or other species are the most widely used genes, with many others under consideration.

The HIV-LTR is strongly activated by the HIV transactivator protein TAT. Therefore, it has been used in conjunction with marker genes as a sensor to detect replication of HIV (Means et al., 1997), and for HIV infection-specific gene expression. Conditional expression of TK driven by the HIV-LTR has been shown to confer to infected cells a rather specific susceptibility to Ganciclovir mediated toxicity (Caruso and Bank, 1997; Miyake et al., 2001). Transfer of TK via a HIV-based lentiviral vector was used to eliminate CD4 tumor cells from adult T-cell leukemia, which is caused by HTLV-I (Obaru et al., 1996). A further possibility would be conditional expression of a toxin. This approach does not require a prodrug, but it is essential that undesired expression, in the absence of the inducing stimulus, does not occur under any circumstance.
5.4 HIV-directed preclinical and clinical studies

Due to the tropism of HIV for cells of the immune system, but also reflecting funding resources, most of T-cell directed vector- and nucleic acid-based antiviral therapies have been aimed at HIV (Gilboa and Smith, 1994). These have included transfer of antisense oligonucleotides, ribozymes or siRNA targeted at viral sequences or to host factors critical for virus replication, e.g. the HIV coreceptor CCR-5; expression of TAR or REV decoy RNAs; expression of intrabodies directed against TAT, REV; expression of transdominant Rev-mutants such as RevM10, and targeting of HIV-coreceptors (summarized in Rössig and Brenner, 2004).

Stimulation of T-cells for efficient transduction can lead to unwanted increases in HIV replication. Further problems with nucleic-acid based therapies (antisense, RNAi, ribozymes) arise from the high mutation rate and enormous sequence variability of HIV-Quasispecies, which contrasts with the high sequence specificity of e.g. RNAi and ribozymes. It is predictable that resistant HIV-1 variants will arise rapidly in vivo. Selection of conserved sequences in the LTR or within leader sequences may partially avoid this evasion mechanism, as will simultaneously targeting of multiple essential HIV-1 sequences or of targeting essential host genes that do not mutate. RNA decoy requires overexpression of TAR- or RRE-Sequences (e.g. by Pol-III-transcription) but is also less susceptible to sequence variation, since it targets viral proteins. Ribozymes have enzymatic activity and are therefore only needed in smaller amounts than classical antisense strategies, though the natural mechanism of RNAi seems to require even smaller amounts of the therapeutic RNA-molecules (reviewed by Peracchi, 2004).

The success of highly active antiretroviral therapy (HAART) with conventional drugs has largely decreased the demand for such novel therapies as a first line approach; however, there may be a place as a supplement, e.g. for transient salvage therapy to eliminate multi-resistant HIV by RNAi.

6. ANTIVIRAL IMMUNOTHERAPY

In the recent years, a valuable addition to conventional tumor therapy has been brought about by specific monoclonal antibodies. These reagents are directed either against antigens preferentially or exclusively expressed on tumors, such as Her2, or in other cases serve to eliminate specific cell populations, that include the tumor cells but also normal cells, such as anti-CD20 (Rituximab). Although responses could not be achieved in all patients with
tumors expressing high levels of the respective target antigens, a focus of attention has been created toward targeted therapeutics, to develop alternative and potentially more efficient reagents.

Cytotoxic T-cell immunity is central to the control of viral infection. T lymphocytes physiologically recognize antigens through TCR interaction with short peptides presented by MHC class I or II molecules. Activation of naïve T cells depends on professional antigen-presenting cells (APCs) that provide necessary co-stimulatory signals for initial and clonal expansion. Without co-stimulation, unresponsiveness and clonal anergy may be the outcome of TCR activation. Furthermore, virus-specific cytotoxic T-cells can not be derived from immunologically naïve individuals.

A variety of techniques to bypass the need for immunization for the generation of immune effector cells with desired recognition specificity have been worked out and will be described below. However, large scale cultivation of (transfected or transduced) T-cells is laborious and technically challenging, as is expansion of virus-specific T-cells when and starting from small number of CTL in the periphery; complex and time consuming protocols for enrichment are necessary to achieve the quantities (~10^7 cells) that are considered to be required for therapy.

6.1 (Vector-mediated) Expansion of antiviral CTL

Adoptive T-cell therapy has been successfully applied to tumors, including EBV-related post-transplant lymphoproliferative disorders (PTLD) (Papadopoulos et al., 1994; Rooney et al., 1995, 1998), and to Cytomegalovirus (CMV)-related disease in immune suppressed transplant recipients (Walter et al., 1995; Peggs et al., 2003; Einsele and Hebart, 2004). Adoptive transfer of gene-marked tumor and HIV-specific CTL has shown intact homing to sites of HIV-replication and local function, as well as good persistence, although therapeutic benefits were limited (Brodie et al., 1999, 2000).

While effective treatment options exist for EBV-related PTLD in the form of Yttrium-90-Labeled Ibritumomab Tiuxetan radioimmunotherapy or Rituximab Immunotherapy (Jaeger et al., 2005; Milpied et al., 2000), CMV-related disease is still highly problematic; CMV reactivation can cause severe morbidity and mortality in immune compromised patients and those with HIV infection, even with appropriate antiviral drug treatment. The constellation of CMV-seronegative donor/CMV-seropositive recipient in allogenic hematopoietic progenitor cell transplantation is at particular risk. In such patients, the recovery of CMV-specific cytotoxic T lymphocytes (CTL) seems to be vital in the reconstitution of CMV specific immunity. Remarkably, beneficial effects were shown for adoptive transfer of
CMV-specific T cell clones or polyclonal CTL from CMV seropositive donors that were expanded \textit{in vitro} (reviewed in Einsele and Hebart, 2004).

### 6.2 Expression of Virus-specific TCRs

Cytolytic effector cells can be genetically modified to carry virus-specific TCR or chimeric receptors based on antibody recognition sites on the surface: Antibody-mediated recognition of viral antigens can thereby be linked with cytolytic effector cells, which then possibly have potential for improved localization, homing and enhanced efficacy.

Transfer of tumor-specific TCRα and β-chains (or their specificity determining regions) to effector T-cells has been done in murine models (Kessels \textit{et al.}, 2001, 2005; Willemsen \textit{et al.}, 2003; Tahara \textit{et al.}, 2003) and can probably be also achieved for virus-specific TCRs. Such transferred receptors will recognize processed peptides that can be derived from diverse viral proteins, not restricted to molecules expressed at the cell surface. However, recognition of MHC-presented peptides will depend on the correct HLA background. The formation of heterodimeric TCR consisting of one original and one transferred TCR chain could in theory give rise to new, potentially autoreactive T-cell specificities.

### 6.3 Directing immune response to viral or infection-specific cellular proteins at the cell surface

Strategies have been developed to specifically target activated T-cells towards infected cells that circumvent MHC-restriction of antigen recognition. The immunoreceptor (“T-body”) strategy relies on transfer of cytolytic T-cells that carry an antigen-specific, recombinant receptor with antigen at the cell surface. In principle, immunoreceptor grafted T-cells can be directed against every cell defined by the particular target signaling properties at their surface. In principle, immunoreceptor grafted T-cells can be directed against every cell defined by the particular target antigen at the cell surface.

Construction of chimeric TCR can be done via gene fusion in which a specific extracellular domain derived from a monoclonal antibody is combined with signal transducing components from T-cells. The specific complementarity determining regions (CDR) of the antibodies variable heavy and light chain, i.e. a single-chain antibody fragment (scFv), mediates recognition of target cells. This is coupled via a transmembrane domain to specific signaling domains of T-cell signal transducing molecules such as the TCRζ-chain and the costimulatory molecule CD28 (Abken \textit{et al.}, 2002). Similar chimeric receptors have been derived from binding domains of natural ligands or antibodies fused directly to the TCRα and TCRβ chains, although this may result in formation of heterodimeric TCR when expressed
on T-lymphocytes. Upon antigen binding, the chimeric receptors generate activating signals in the effector cell similar to those initiated by the TCR complex.

Delivery of such recombinant molecules into NK- or T-cells with cytolytic capacity has been shown to direct them toward the target which is specifically recognized by the antibodies CDR. The major advantage of this approach compared to expression of a conventional TCR is that recognition is not restricted to HLA, which would allow preparation, expansion and storage of virus specific cytotoxic cells for use “of-the-shelf”. Furthermore, presentation of a peptide by MHC is not required. This is specifically interesting as a number of viruses, including human CMV, are able to downmodulate MHC from the surface of infected cells (Mocarski, Jr., 2004; Hewitt, 2003). On the other hand, the target molecule has to be presented on the surface of the infected cell (or tumor cell) in a form that is recognizable for the antibody CDR. Processed peptides derived from target molecules that are presented on the MHC are usually not recognized.

In initial clinical studies, infusion of such cells into patients proved to be safe and transient therapeutic effects have been observed. Initial HIV-directed clinical trials lacked therapeutic effectiveness and persistence of modified cells in vivo. There are inherent limitations of the immunoreceptor technology: Impaired persistence may result from recognition of the chimeric receptor as foreign, resulting in elimination. Furthermore, T-helper cells are required for efficient CTL function. Impaired signaling capacity that does not achieve the appropriate signal strength in the right context, and lack corresponding costimulatory signals, could also lead to anergy or elimination. Enhanced receptor molecules have therefore been constructed that include functional domains from costimulatory molecules. The promising technique could also be improved by usage of humanized molecules, as well as transduction of cells with specificity against a strong antigen, such as EBV, to improve persistence (summarized in Rössig and Brenner, 2003, 2004).

6.4 Problems arising from performance enhanced immune cells

Graft versus host disease like effects resulting from autoaggressive behavior of transferred autologous CTL have been observed in other contexts. The altered specificity of T-bodies and heterodimeric TCR can possibly induce autoimmune side effects, though they have not been observed with the relatively small patient numbers treated to date. Lymphoproliferative syndrome can be caused by transduced cells that result from insertion mutagenesis by the vector; this danger might be partially overcome by self-
inactivating vectors, by introduction of suicide genes to ablate the unwanted cells, or by gene transfer into mature cells that may be easier to eliminate than stem cells. Possible solutions could be preselection of transferred cells for integration sites, if suitable strategies can be developed. Immunogenicity or undesired behavior of suicide genes could be overcome by novel physiological suicide genes such as CD20 (for elimination by Rituximab, Introna et al., 2000), or conditional proapoptotic molecules derived from endogenous genes (summarized in Rössig and Brenner, 2004).

7. VECTOR- AND NUCLEIC ACID-BASED VACCINATION STRATEGIES

Numerous vector-based vaccination and immunity enhancing strategies have been developed, also employing specific gene transfer into professional APC such as Langerhans or dendritic cells (DC) (Jenne et al., 2001). RNA- and DNA-virus-based replicons that are under development for prophylactic and therapeutic vaccination are mostly targeted against HIV, viral hepatitis, HPV-associated cervical cancer (Chapter 2.5 by L. Gissmann), as well as toward more fashionable and recent threats such as SARS-CoV, Filovirus, and those relating to “Biodefense” (Lee et al., 2005). The reader is referred to recent reviews focusing on Adenovirus- (Tatsis and Ertl, 2004) Alphavirus- (Lundstrom, 2001), and DNA-vaccine vectors (Srivastava and Liu, 2003; Duenas-Carrera, 2004; Giri et al., 2004). Vaccines based on Adenoviruses or on gene-modified poxviruses (Drexler et al., 2004; Moss, 1996), such as modified Vaccinia strain Ankara (MVA), may be specifically considered in situations where adequate medical supply chains and financial resources do not exist. Vaccine formulations based on (lyophilized) DNA are also highly stable under adverse circumstances. The success of Variola elimination did crucially depend on the enormous stability, effectiveness and low cost of the Vaccinia virus. MVA-based vaccination trials showed good safety and immunogenicity of MVA expressing HIV Nef in HIV patients (Harrer et al., 2005), and there is promising data on regression of precancerous cervical lesions in a preclinical study of MVA expressing HPV E2 (Corona Gutierrez et al., 2004).
CONCLUSIONS

The intensified use of genetically modified viruses as gene transfer vectors has to consider a number of potential risk factors and their implications for activities with viral vectors. Specific and careful risk assessment is necessary from the perspective of naturally occurring cross-species transfer of viruses, and of possible host-range mutants resulting either from cell culture (using non-host species cells) or tropism engineering (Louz et al., 2005). It remains open if viral vector-based antiviral therapy can be curative in an already established persistent infection. Yet such strategies using gene modified cells may be useful to (transiently) support the host immune system in its struggle with the viral intruder until its own resources have sufficiently recovered. The challenge will be to maintain the balance.

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