CHAPTER I

INTRODUCTION

1. Gene therapy

Gene therapy is a methodology for correcting defective genes responsible for disease development. Two methods are available for inserting genetic material into human chromosomes. The first, called the *ex vivo* technique, involves surgically removing cells from the patients, injecting or splicing the new DNA (the DNA that will correct the disease) into the cells and letting them divide in cultures. The new tissues are placed back into the affected area of the patient.

The second method is called *in vivo* technique that consist in the direct injection of therapeutic DNA into the body cells. A carrier molecule called vector must be used to deliver the therapeutic gene to the patient's target cells. Currently, the most common vectors are viruses that have been genetically altered to carry normal human DNA. Viruses have evolved a way of encapsulating and delivering their genes to human cells in a pathogenic manner. Scientists have take advantage of this capability and manipulated the virus genome to remove disease-causing genes and insert therapeutic genes. Target cells such as the patient's liver or lung cells are infected with viral vectors. The vectors then unload their genetic material containing the therapeutic human gene into the target cells. The generation of a functional protein product from the therapeutic gene restores the target cell to a normal state. Some of the different types of viruses used as gene therapy vectors:

- Retroviruses
- Adenoviruses
- Adeno-associated viruses

Besides virus-mediated gene-delivery systems, there are several nonviral options for gene delivery. The simplest method is the direct introduction of therapeutic DNA into target cells. This approach is limited in its application because it can be used only with certain tissues and requires large amounts of DNA.

Another nonviral approach involves the creation of an artificial lipid sphere with an aqueous core. The liposomes, which carry therapeutic DNA, are capable of passing the DNA through the target cell's membrane. Therapeutic DNA also can be introduced in target cells by chemically linking the DNA to a molecule that will bind to special cell receptors. Once bound to these receptors, the therapeutic DNA constructs are engulfed by the cell membrane and passed into the interior of the target cell. This delivery system tends to be less effective than other options.

Gene therapy includes some of problems that the scientists are trying to solve:

1) Short-lived duration of gene therapeutic approch: before gene therapy can become a permanent cure for any condition, the therapeutic DNA introduced into target cells must remain functional and the cells containing the therapeutic DNA must be long-lived and stable. Problems with integrating therapeutic DNA into the genome and the rapidly dividing nature of many cells prevent gene therapy from achieving long-term benefits. At the moment patients should undergo multiple rounds of gene therapy.

2) Immune response: anytime a foreign object is introduced into human tissues, the immune system is designed to attack the invader. The risk of stimulating the immune system in a way that reduces gene therapy effectiveness is always a possibility.

3) Multigene disorders: conditions or disorders that arise from mutations in a single gene are the best candidates for gene therapy. Unfortunately, some of the most commonly occurring disorders, such a heart disease, high blood pressure, Alzheimer's disease, arthritis, and diabetes, are caused by the combined effects of variations in many genes. Multigene or multifactorial disorders such as these would be especially difficult to treat effectively using gene therapy.

4) Chance of inducing a tumor (insertional mutagenesis): if the DNA is integrated in the wrong place in the genome, for example in a tumor suppressor gene, it could induce a tumor. This has occurred in clinical trials for X-linked severe combined immunodeficiency (X-SCID) patients, in which hematopoietic stem cells were transduced with a corrective transgene using a retrovirus, and this led to the development of T cell leukemia in 3 of 20 patients.

2. Biology and viral life cycle of adenovirus

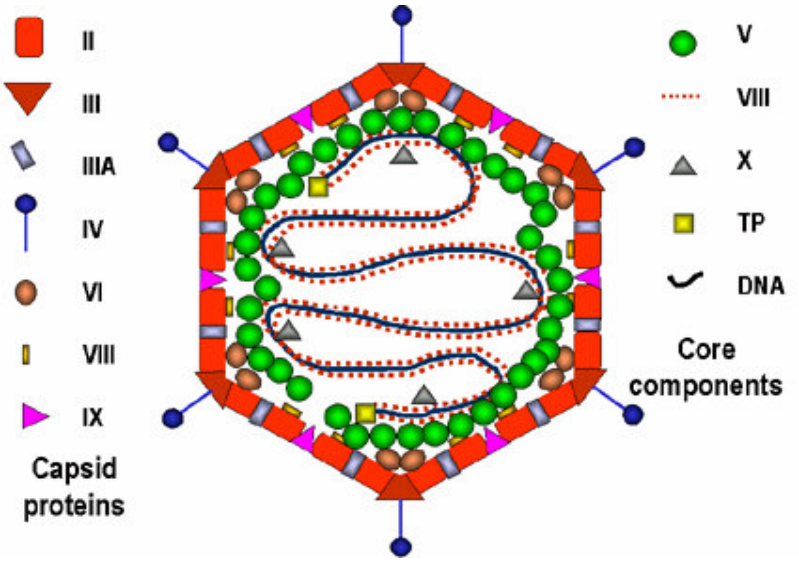
2.1 Description and general properties of adenoviruses

Adenoviruses were first discovered half a century ago by Rowe and colleagues, who were trying to culture adenoid tissue in the laboratory (Rowe et al., 1953). Non human adenoviruses have been isolated from a number of species including chimpanzees, pig, mouse, dog, and other mammalian and avian species (Shenk, 1996). Although human adenoviruses cause significant levels of respiratory, ocular and gastrointestinal disease, they have been the object of intense study over the years mainly as a model system for basic eukaryotic cellular processes such as transcription, RNA processing, DNA replication, translation and oncogenesis. Currently, adenoviruses are a popular choice as a gene delivery vehicle and in all gene therapy clinical trials are second only to the use of retroviral vectors.

Adenoviruses belong to family of Adenoviridae; currently, the 51 serotypes of human adenovirus are divided are in six groups (A to F) based on sequence homology and their ability to agglutinate red blood cells (Shenk, 1996). The adenovirus genome is a double-stranded, 36-kb approximately long linear DNA. Each end of the genome has an inverted terminal repeat (ITR) of 100-140 bp to which the terminal protein (TP) is covalently linked. Adenoviruses have a characteristic morphology (Stewart et al, 1993). The capsid, a proteic structure that covers the adenovirus genome is icosahedral. The capsid vertices consist of the penton base, which acts to anchor the fibre protein, responsible for primary attachment of virions to the cell surface. The facets of the virus capsid are composed primarily of trimers of the hexon protein, as well as a number of other minor components including protein pIIIa, pVI, pVIII and pIX ***(Fig. 1)***. The protein VII, a small peptide termed *mu* (Anderson et al., 1989) is intimately associated with the virus DNA, while the protein V, is packaged with this DNA-protein complex and appears to provide a structural link to the capsid via protein VI (Matthews and Russel, 1995). Members of adenovirus family infect a great variety of post-mitotic cells, even those associated with highly differentiated tissues such as skeletal muscle, lung, brain, and heart. Since they deliver their

genome to the nucleus and can replicate with high efficiency, they are prime candidates for the expression and delivery of therapeutic gene and have a wide host-range.

A



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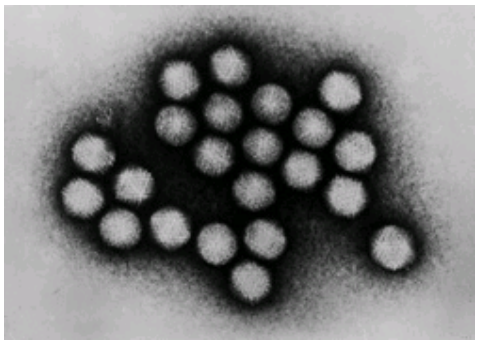


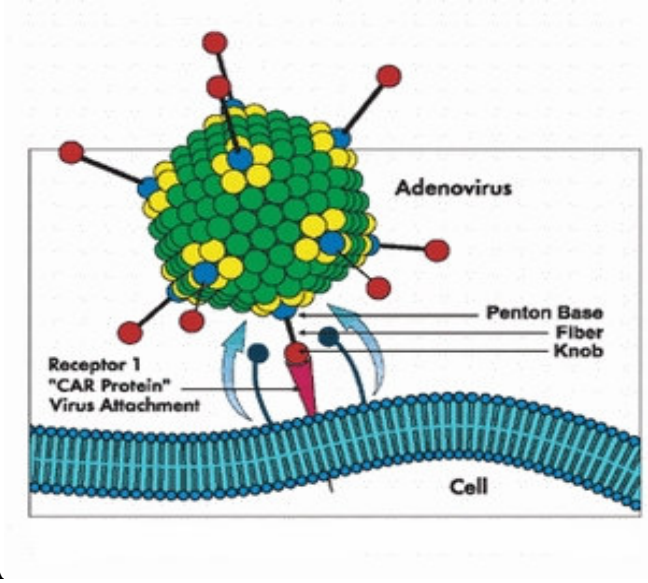
Figure 1 Adenovirus structure: A) A stylized section of the adenovirus particle based on current understanding of its polypeptide components and DNA. No real section of the icosahedral virion would contain all the components. Virion constituents are designated by their polypeptide numbers with the exception of the terminal protein (TP). B) Adenovirus seen through electron microscopy.

2.2 The viral life cycle

2.2.1 Binding and entry

For all groups, except group B adenoviruses, initial attachment of virion particles to the cell surface occurs through binding of the fibre Knob to the receptor that is identical to that for Coxsackie B virus (Bergelson *et al.,* 1997) and has therefore been termed the coxsackie/adenovirus receptor (CAR). This is a plasma membrane protein of 46 kDa belonging to the immunoglobulin superfamily and contains extracellular, transmembrane and cytoplasmic domains (Tomko *et al.,* 1997), with extracellular domain being sufficient for attachment. (Wang and Bergelson, 1999). CAR normally functions as a cell-to-cell adhesion molecule on the basolateral surface of epithelial cells (Honda *et al*., 2000). The CD46 molecule, a complement- regulatory protein, has been identified as a cellular receptor for group B adenoviruses (Gaggar *et al.,* 2003).

After initial attachment ***(Fig.2)*** to the cell surface, an exposed RGD motif on the penton base interacts with member of αv integrin family, triggering virus internalization by clathrin- dependent, receptor-mediated endocytosis (Stewart *et al.,* 1997; Meier *et al.,* 2000) Integrins form a large family of heterodimeric receptors and it appears that integrins αvβ3 e αvβ5 both support adenovirus internalization αvβ5 is expressed on human bronchial epithelial cell, a major site of primary adenovirus infection *in vivo* (Mette *et al.,* 1993). Interaction of the virus with plasma membrane can induce a number of signalling pathways such as the activation of the phosphoinositide-3-OH kinase (PI-3k) pathway, which in turn triggers the Rho family of GTPases and the polymerization and reorganization of actin to facilitate endocytosis (Li *et al.,* 1998; Rauma *et al.,* 1999).



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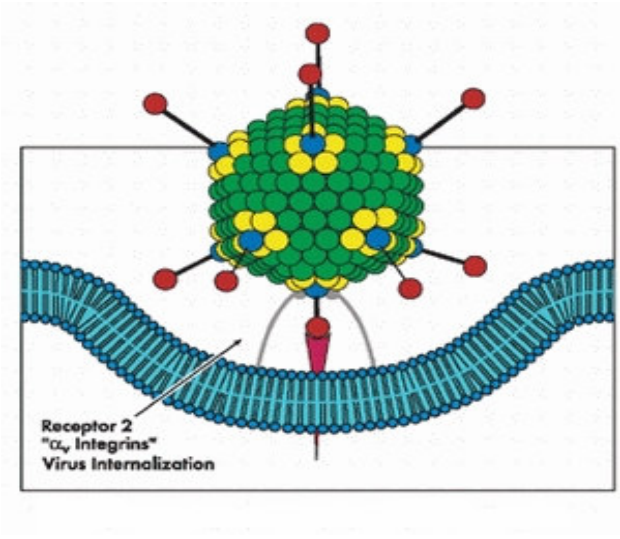


B

Figure 2 Binding and internalization of Adenovirus: A) the adsorption of the virus to target cell receptors involves high-affinity binding via the knob portion of the fibre. The prime receptor for human Adenovirus serotype 5 is identical to that for coxsackie B virus and has been named the Coxsackie/Adenovirus receptor (CAR). After the attachment step, B) interaction between the penton base and αv integrins on the cell surface leads to internalisation of the virus through endocytosis.

As early as 20 min post-infection, activation of Raf/mitogen- activated protein Kinase (MAPK) pathway and consequential production of IL-8 have been observed (Bruder and Kovesdi, 1997).

For Ad2 and Ad5, the acidic environment of the endosome induces escape of virions into the cytoplasm, although the mechanisms underlying this process are poorly understood. Once in the cytoplasm ***(Fig.3)***, dynein mediates trafficking of virions along microtubules toward the nucleus, where they subsequently dock with the nuclear pore complex (NPC) (Trotman *et al*., 2001; Kelkar *et al.,* 2004). Disassembly of the capsid at NPC allows for import of the viral genome and commencement of the viral transcriptional program.

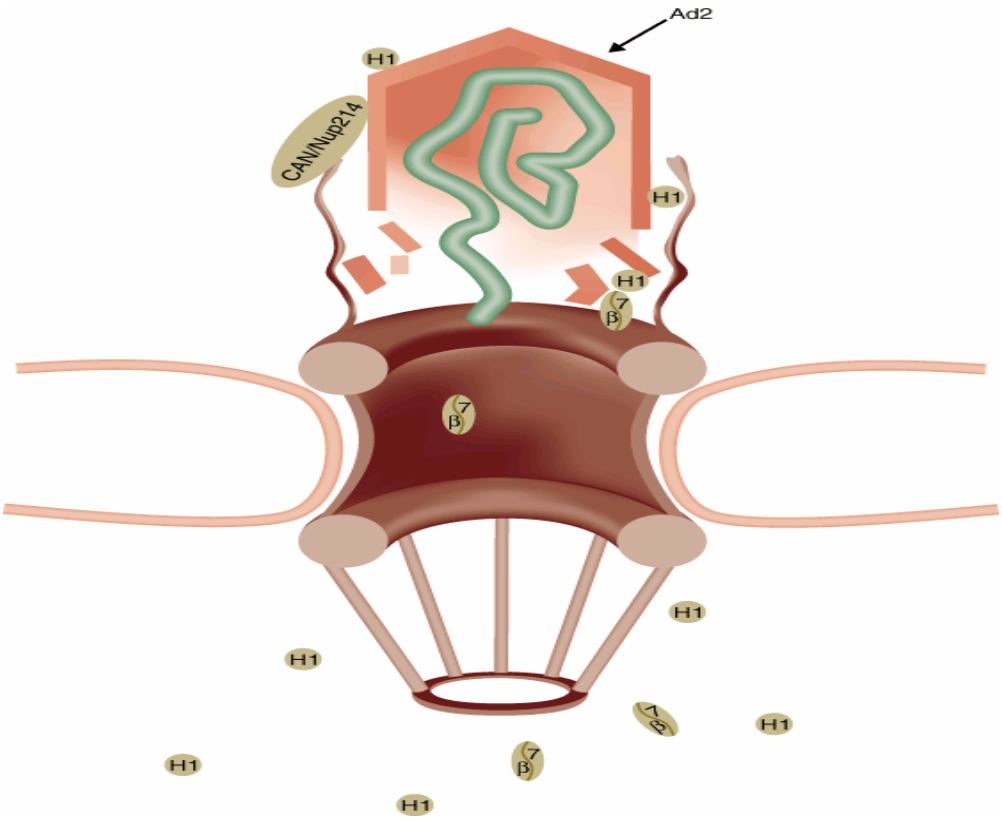


Figure 3 Model for nuclear entry of Ad2: The Ad2 particle docks on the cytoplasmic fibrils of the nuclear pore by binding CAN/Nup214. Small amounts of histone H1 escape from the nucleus and bind to hexon protein on the proximal side of the docked capsid. Importin β–importin 7 dimers bind to H1, inducing import of the proximal H1–hexon complexes and triggering capsid disassembly. Consequently, the viral DNA is liberated near the opening of the pore and positioned for translocation into the nucleus.

2.2.2 Early genes and DNA replication

The adenovirus infectious cycle can be defined into two phases: the "early" phase and the "late" phase, respectively occurring before and after virus DNA replication ***(Fig. 4)***. The first phase covers the entry of the virus into the host cell and the passage of the virus genome to the nucleus, followed by the selective transcription and translation of the early genes. These early events modulate the functions of the cell so as to facilitate the replication of the virus DNA and the resultant transcription and translation of the late genes. This leads to assembly in the nucleus of the structural proteins and the maturation of infectious virus. The early phase in a permissive cell can take about 6-8 h (depending on a number of extraneous factors), while the late phase is normally much more rapid, yielding virus in another 4-6 h.

The first viral transcription unit to be expressed is E1A that produces multiple mRNA and protein products by way of differential mRNA processing. Two E1A transcripts are produced during early infection: 13S mRNA encoding the 289R (where R stands for amino acid residues) protein in (Ad5) and 12S mRNA encoding the 243R. These proteins can immortalize primary cells in culture and, when expressed in conjunction with E1B proteins, cause tumours in rodents (reviewed by Ben- Israel and Kleinberger, 2002). During infection, the E1A proteins function to *trans*-activate the other adenovirus early transcription units (E1B, E2, E3 and E4) and to induce the cell to enter S phase in order to create an environment optimal for virus replication (Berk, 1986).

The E1A proteins have been shown to use a variety of mechanisms to subvert cell cycle checkpoints ; E1A can directly bind and inhibit components involved in cell cycle control such as the cyclin-dependent kinase inhibitor p21 (Chatopadhyay *et al.,* 2001). Furthermore, E1A can interact with a number of host factors involved in mediating chromatin structure including p400 (Fuchs *et al.,* 2001) and the histone acetyltransferases (HATs) p300/CBP, p CAF and TRRAP/GCN5 (Lang and Hearing, 2003).

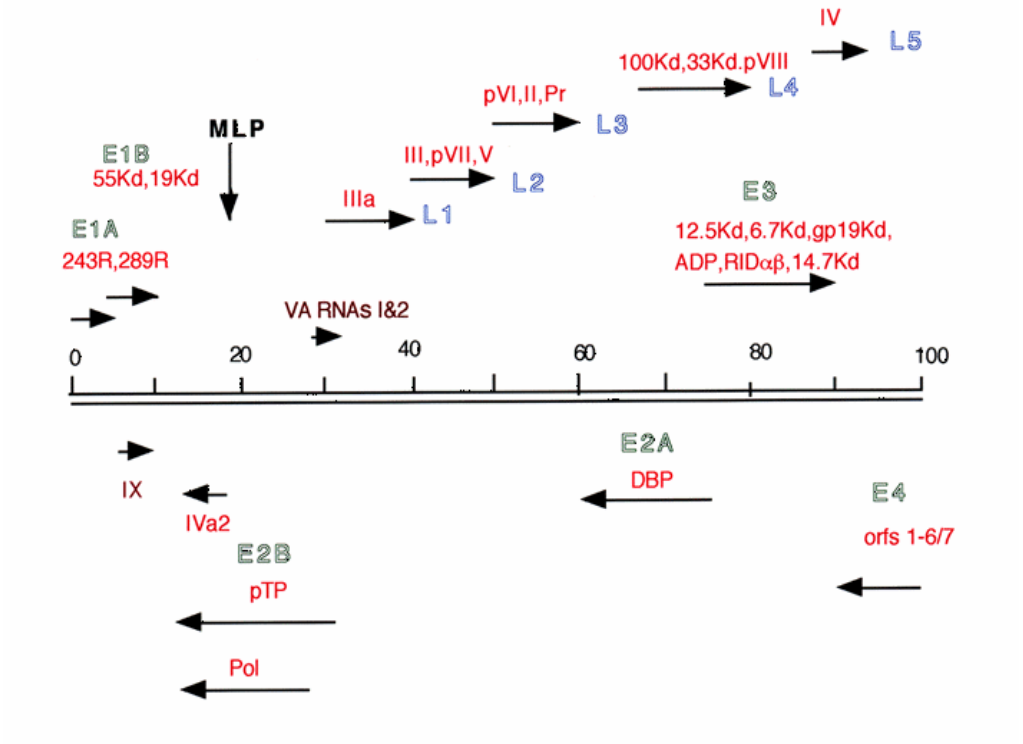


Figure 4 Transcription of the adenovirus genome: The early transcripts are outlined in green, the late in blue. Arrows indicate the direction of transcription. The gene locations of the VA RNAs are denoted in brown. MLP, Major late promoter.

Cell cycle deregulation by E1A results in accumulation of the tumour suppressor p53 and expression of E1A during infection promotes apoptosis by sensitizing cells to the tumour necrosis factor α (TNF- α) and TRAIL (TNF- related apoptosis-inducing ligand)-mediated death receptor pathways (Routes et al., 2000). The E1B 19K product is able to block downstream mediators of these pathways and inhibit programmed cell death (Perez and White, 2000) In the case of TNF- α mediated apoptosis, the E1B-19k protein can bind directly to the proapoptotic proteins Bak and Bax to prevent mitochondria-mediated apoptosis (Sundararajan et al., 2001). In addiction to its antiapoptotic functions, the E1B-55K protein facilitates the transport of viral mRNAs to the cytoplasm during the late stages of infection (Pilder et al., 1986).

The E2 region encodes proteins necessary for replication of the viral genome: DNA polymerase, preterminal protein, and 72-kDa single-stranded DNA-binding protein. These provide the machinery for replication of virus DNA action and ensuing transcription of late genes and this is mediated by interaction with a number of cellular factors. Products of the viral E3 region, which are dispensable for the replication of virus in tissue culture, function to subvert the host immune response. The immune system has evolved a number of mechanisms for destroying virus-infected cells, including cell lysis by cytotoxic T lymphocytes and activation of receptor- mediated apoptotic pathways by chemokines. The E3-gp19k protein act in two ways to prevent the presentation of viral antigens by the MHC class I pathway and subsequent cell lysis by cytotoxic T cells. E3-gp19k was first found to prevent translocation of MHC class I molecules to the cell surface by sequestering them in the endoplasmatic reticulum. More recently, it has been shown that E3-gp19k can bind to TAP (transporter associated with antigen processing), an ER protein responsible for transporting cytosolic antigens into the lumen, suggesting that the E3-gp19k protein may directly interfere with the loading of peptides onto MHC class I molecules (Bennet et al 1999). The E3-10.4K, 14.5K and 14.7K proteins have all be shown to inhibit the induction of apoptosis by the chemokines TNF-α, Fas ligand (FasL), and TRAIL.

The E4 transcription unit encodes a number of proteins (termed orfs 1-6/7) that have been known to play a role in cell cycle control and transformation. E4orf1

protein of Ad9 demonstrated that it is able to induce estrogens-dependent mammary tumours in mice (Javier et al., 1991). In Ad2 and Ad5, E4orf3 and E4orf6 encode gene products with a number of diverse functions. Both proteins have been shown to increase the ability of E1 genes to transform primary rodent cells, increase the expression of viral late genes and inhibit genome concatemerization by cellular DNA repair enzymes (Stracker et al., 2002). In the case of E4orf6, enhanced transformation is thought to occur via its ability to block p53-mediated *trans*-activation by inhibiting the binding of p53 cellular transcription factors (Dobner et al., 1996). In addition to the function listed, E4orf3 protein also mediates the organization of nuclear structures termed PML oncogenic domains. Although the function of these domains is not clear they have been shown to play a role in transformation, transcription, and apoptosis in infected cells (reviewed by Maul, 1998). Most products of the E4 region have antiapoptotic effects; however, the E4orf4 interacts with protein phosphatase 2A to stimulate p53 independent apoptosis (Shtrichman et al., 1999)

2.2.3 Late gene expression and viral assembly

The major late promoter (MLP) transcribes adenovirus late genes that are expressed from five regions, L1-L5. The major late transcription unit (MLTU) encodes approximately 15 to 20 different mRNAs, all of which are derived from a single pre-m RNA by differential splicing and polyadenylation. These transcripts primarily encode structural proteins of the virus and other proteins involved in virion assembly. After the onset of DNA replication, transcription from the MLP is induced to high levels, ensuring the production of adequate amounts of structural proteins for the assembly of progeny virions. Manipulation of late genes encoding the structural components of the capsid has been explored as a strategy for changing the tropism of gene therapy vectors. The L1-52/55k protein is required for the encapsidation process, while L4-33K protein also appears to play a role in virus assembly as mutants carrying complete or partial deletions of this gene are defective in capsid formation (Finnen et al., 2001).

The packaging sequence itself is a series of seven repeats (A1-A7) and the left end of the genome (Hearing et al., 1987). Although each of the repeats fits a

consensus motif, they are nay functionally equivalent as A1, A2, A5 and A6 have been shown to be most important for genome encapsidation (Grable and Hearing, 1990). Once assembly and DNA encapsidation have occurred, the adenovirus protease cleaves a subset of the structural proteins into their mature form to produce fully infectious virions (reviewed by Mangel et al., 2003). Cell lysis and release of progeny virions occur approximately 30hr post infection in a process involving the E3-11.6K protein, also called the adenovirus death protein (ADP); (Tollefson et al 1996a). Unlike other products of the E3 region, ADP is produced only during the late phase of infection and is transcribed from the MLP rather than E3 promoter (Tollefson et al 1996b).

3. Adenoviruses as vectors

Adenoviruses can infect a wide variety of cell types and tissues in both dividing and non-dividing cells. This characteristic, together with their relative ease of preparation and purification, has led to their extensive use as a gene vectors.

Vectors *(Table 1)* can be utilized for: 1) cancer therapy to deliver genes that will lead to tumour suppression and elimination; 2) gene therapy 3) supplementary therapy to deliver genes, expression of which will combat disease process.

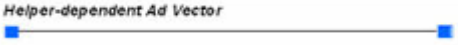
Adenovirus, Adenoviral Vector	Capacity	Vector Replication	Vector Production
	-	Yes	No additional component other than producer cell needed
	4 kb	Yes	No additional component other than producer cell needed
	8 kb	No	Expression of AdE1 in producer cell needed
	36 kb	No	Helper vector needed for production

Table 1 General properties of adenoviral vectors

3.1 First-generation vectors

In the first generation of vector, the E1 region necessary for activation of viral promoters and expression of both early and late genes, were removed and so this viruses are severely impaired in their ability to replicate. For these reasons, replacement of the E1 region with transgenes was the initial strategy used in construction of adenoviral vectors, giving rise to the so-called first-generation vectors. The ability to delete E1 region is made possible by the existence of cell lines that provide these functions *in trans.* The classic cell line for this purpose is the 293 cell line, a human embryonic kidney-derived line that has been transformed by the adenovirus E1 region (Graham *et al.,* 1977) Production of E1 deleted vectors was initially carried out by homologous recombination in

mammalian cells between constructs carrying the left and right ends of the genome (Chinnadurai *et al.,* 1979). Removal of the E1 region alone allows approximately 5.1 kb for insertion of therapeutic genes because adenovirus can package up 38 kb without affecting viral titer and growth rate (Bett et al., 1993).

Many of the first-generation vectors also contain a deletion in the E3 region, mainly for practical reasons. To optimize the yield of vectors in early experiments using overlap recombination, investigators used the Ad type 5 mutant dl309 or its derivates, which contain in the E1 region two unique restriction sites due to partial deletion of E3 (Jones and Shenk 1978).

Thus, the likelihood of regeneration of the starting wild-type virus, which could arise as a result either of incomplete restriction digestion or religation of viral DNA in the cell, was minimized. Furthermore, E3 genes are entirely dispensable for virus growth in vitro and their removal, together with deletion of E1 genes, allows up to 8.2 kb for transgenes insertion. Data have suggested that expression of E3 genes from vectors may be beneficial in vivo because of their ability to dampen many host immune processes. It has been reported that expression of the entire E3 region or the E3-gp19K product alone can increase persistence of transgene expression in some rodent models (Ilan et al., 1997). However, conflicting data have shown that expression of the E3-gp19K protein has no effect on the length of transgene expression (Schowalter et al., 1997). These discrepancies may be due in part to differences in the nature of the transgene or the tissue type that was analyzed. Nevertheless, the inclusion of E3 genes in vectors remains an area of active investigation.

Although first-generation vectors have proven to be highly promising as vehicles for gene delivery, problems do exist. The first drawback associated with these vectors becomes apparent during vector production. Recombination between the E1 region sequences in the complementing cell line and the recombinant virus can give rise to viral progeny with functional E1 genes that are replication competent (Lochmuller et al., 1994).

Thus, recombinant virus stocks must be assayed for the presence of replication-competent viruses. Helper cell lines such as PERC6 and 911, in which the overlap between E1 sequences in the cell and those commonly present on recombinant

virus chromosomes is reduced, have been constructed in order to minimize this occurrence . The second and more troublesome problem associated with the use of first generation vectors is their stimulation of a cellular immune response, resulting in the destruction of transduced cells that are expressing therapeutic transgenes. Indeed, a number of early studies showed that administration of E1-deleted vectors to immune-competent animals results in only transient transgene expression (Dai et al., 1995). It is theorized that the immune response is stimulated by low levels of replication that can occur even in the absence of the E1 genes. This idea is supported by findings that genome replication and late gene expression can occur from E1-deleted vectors in vivo (Yang et al., 1994a, b). Although stimulation of a robust immune response may preclude the use of first-generation vectors in some settings, they remain promising for applications requiring short-term gene expression such as cancer therapy and vaccination.

3.2 Second-generation vectors

To prevent the immune response generated by low-level replication of E1-deleted viruses, vectors deleted for multiple genes have been created to inhibit viral gene expression more effectively. These second-generation vectors have been constructed primarily by the removal of E2 and E4 coding sequences, also providing the benefit of a larger capacity for transgene insertion. The major drawback encountered during construction of these multiply deleted viruses is the need for isolation of cell lines expressing the missing functions *in trans*. Although this can be a time-consuming process, vectors propagated in these cells are less likely to undergo recombination to give replication-competent viruses. In the case of E2 genes, cell lines have been produced that stably express the single stranded DNA-binding protein, preterminal protein, the viral DNA polymerase, or a combination of the three (Amalfitano and Chamberlain, 1997). Vectors containing deletions in these genes are incapable of genome replication, and in the case of polymerase-deficient vectors, no replication occurs even in the presence of high levels of E1A (Amalfitano et al., 1998).

3.3 Helper-dependent vectors

The approach that holds perhaps the most promise for long-term gene expression in the absence of complicating effects due to the presence of viral genes is that of gutted, or helper-dependent, adenovirus vectors (Clemens et al., 1996; Chen et al., 1997). In this strategy, all of the viral structural genes are deleted from the viral chromosome, leaving just the two ITRs and the packaging signal. Such a chromosome can accommodate up to 37 kb of transgene sequences. To propagate the helper-dependent genome, the presence of a helper virus that provides the functions required for replication and assembly is required, as production of a complementing cell line has not been possible because of the need for high levels of some virion components and the toxicity of some of these proteins to the cell. The main problem to date is the inability to completely separate virions containing the helper-dependent chromosome from those containing the helper virus genome (Sandig et al., 2000). Early strategies that were pursued to reduce helper virus contamination included the use of a helper virus carrying a mutated packaging signal, and minimizing the size of the helper-dependent chromosome compared with that of the helper virus with the hope that the two types of virions could be separated based on their different densities. Even using these techniques, however, helper-dependent virus preparations contained significant levels of contaminating helper virus. More recently, helper viruses in which the packaging sequence is flanked by *loxP* or *frt* sites have been constructed (Umana et al., 2001). When these viruses are used to propagate helper-dependent vectors in cells expressing Cre and Flp, respectively, the packaging sequence on the helper virus is excised, resulting in a significantly lower percentage of contaminating helper virus. Indeed, by deriving improved helper cell lines and culture conditions, helper virus levels can be reduced to below 0.01% (Palmer and Ng, 2003).

However, recombination between helper-dependent and helper chromosomes, leading to helper chromosomes that can be packaged, is still encountered during virus propagation. A novel method using baculovirus to provide helper functions was reported to allow for production of helper-dependent vectors without contamination by helper virions, although attempts to use this process for large-

scale preparations also resulted in the formation of replication-competent viruses (Cheshenko et al.,2001). Nevertheless, in vivo studies using helper-dependent vectors have produced promising results (Pastore et al., 2004).

3.4 Induction of innate immune response and toxicity by adenoviral vectors

The primary function of the host immune response to a virus is to detect rapidly, limit and ultimately eradicate an infection. The innate immune system plays a key role as the first line of defense in this process.

The cellular immune response towards adenovirus antigens is activated by antigen-presenting cells (APCs). After the uptake of the Ad particle, viral proteins and transgenes products are processed into small oligopeptides, which are presented by the major histocompatibility complex (MHC) class-I molecules at the cell surface. It is noteworthy that the de novo synthesis of viral proteins does not appear to be required for antigen presentation, since psoralen-treated, UV-cross-linked, inactive adenovirus vectors still cause activation of a cellular immune response (Kafri T, Morgan D, Krahl T, et al. 1998) The binding of CD8+ T cells to this peptide–major histocompatibility complex (MHC) class-I initiates the formation of Ad-specific or transgene-product-specific CTLs ***(Fig.5)***. The interaction between CD28 and B7 plays a co-stimulatory role in this activation (Linsley PS, Brady W, Grosmaire L, et al, 1991). The cellular immune response is further stimulated by CD4+ helper cells primarily belonging to the Th1 subset (Yang Y, Xiang Z, Ertl HC, Wilson JM, 1995). In contrast to the CD8+ T cells, these CD4+ helper cells are activated by epitopes from the input virion, which are presented by MHC class-II molecules at the surface of APCs. This activation triggers the Th1 cells to secrete interleukin-2 (IL-2) and interferon-gamma (IFN-γ). These cytokines, in turn, induce the differentiation of CD8+ T cells into CTLs (Maraskovsky E, Chen WF, Shortman K. 1989) In addition, IFN-γ causes the up regulation of MHC-I expression in Ad-transduced cells and consequently facilitates their recognition by CTLs. Moreover, activated CD4+ helper cells have also been suggested to destroy Ad-transduced cells themselves, resembling in this way primary CTLs (Yang Y, Wilson JM, 1995).

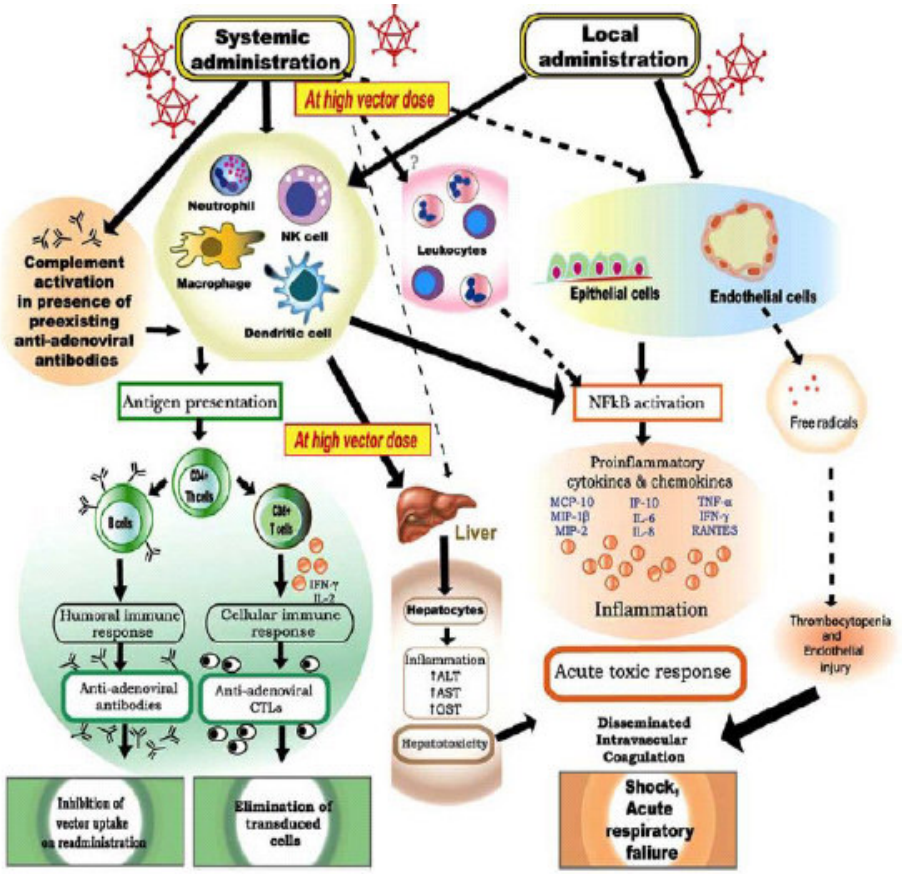


Figure 5 Development of adenoviral (Ad) vector immunity: The first use of an Ad vector leads to a strong innate as well as adaptive immune responses resulting in development of neutralizing antibodies and elimination of transduced cells. In response to high amount of vector administration, a strong innate immune is initiated, which is characterized by production of a variety of proinflammatory cytokines and chemokines leading to an acute toxic response and hepatotoxicity.

Apart from the cellular immune response, the adaptive immune system also includes a humoral component, which constitutes a second hurdle to persistent transgene expression. This humoral immune response is initiated by the binding of adenovirus particles to the surface immunoglobulin of B cells (Janeway CA, Travers P, 1997). After internalization and processing of the virus, the adenovirus derived epitopes are presented at the surface of the B cell by MHC-II molecules. The resulting antigen–MHC-II complex can be recognized by activated T helper cells of the Th2 subset (Paul WE, Seder RA, 1994).

This specific CD4+ helper cell subset releases cytokines, like IL-4, IL-5, IL-6 and IL-10, which provide indispensable signals for the B cells to differentiate into plasma cells. As a result, the plasma cells secrete antibodies (Abs), which are directed towards the adenoviral capsid. Although T helper cells of the Th1 subset are poor initiators of the humoral immune response, they do play a role in Ab-isotype switching ((Boom WH, Liano D, Abbas AK, 1988). Whereas Th2 cells control the production of the Abs isotypes IgG1, IgG2b, IgA and IgE by cytokines, such as IL-4, Th1 cells control the switch to IgG2a or IgG3 by means of IFN-γ secretion (Finkelman FD, Holmes J, Katona IM, et al. 1980). The development of Ad-specific antibodies does not contribute to the elimination of Ad-transduced cells and hence does not affect the persistence of transgene expression. However, Ad-specific Abs will bind the Ad vector and thereby prevent cell entry and promote opsonization by macrophages. Consequently, Ad-specific Abs hampers the efficacy of repeated administrations of the Ad vector, which would be required to keep the transgene expression at the desired level. Thus, although repeated administrations of the Ad vector can prolong expression of the transgene in immunodeficient recipients, the efficiency is dramatically reduced in immunocompetent recipients (Dai Y, Schwarz EM, Gu D, et al. 1995).

Moreover, a large proportion of the human population harbours humoral immunity to Ad vectors because of previous infections (Chirmule N, Propert K, Magosin S, et al, 1999). Consequently, effective adenovirus-mediated gene transfer in humans may be frustrated even at the first administration of an Ad vector ***(Fig.6)***. It is noteworthy that vector-specific immunity does not prevent the

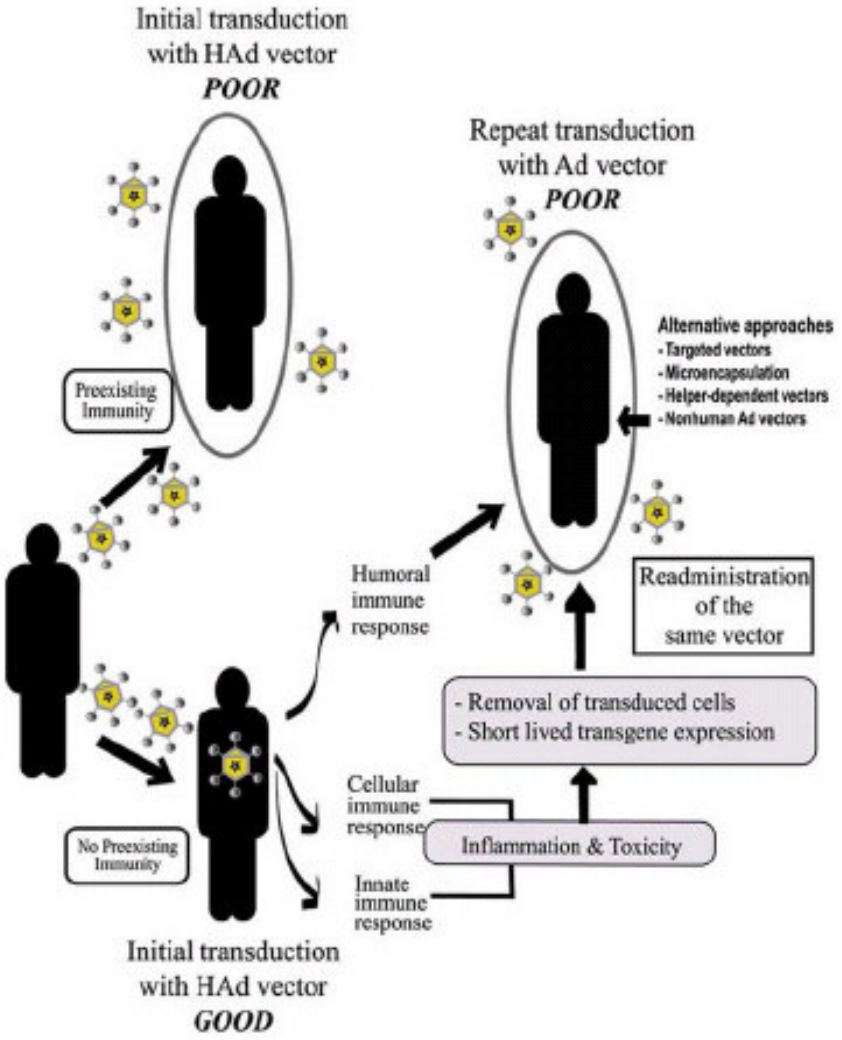


Figure 6 Pre-existing immunity as a barrier to adenoviral (Ad) gene therapy: The presence of pre-existing immunity in the majority of human population interferes with initial transduction with HDAd vectors. In case of individuals with no pre-existing immunity, the first inoculation with an HDAd vector may be successful but subsequent development of strong cellular and humoral immunity renders repeat administration of the same vector less effective.

vector-specific activation of the innate immune system that occurs at very-high vector doses (Varnavski AN, Zhang Y, Schnell M, et al. 2002).

Apart from Ad-specific Abs, neutralizing Abs might also be generated against the transgene product.

This transgene-product-specific Abs can neutralize the transgene product once it enters the circulation and thereby abrogate the effect of gene transfer, irrespectively of persistence of transgene expression (Fields PA, Kowalczyk DW, Arruda VR, et al.2000). Moreover, several studies have indicated that, instead of the immunogenicity of adenoviral proteins, the immunogenicity of foreign transgene-encoded proteins is a primary determinant of the persistence of transgene expression (Morral N, O'Neal W, Zhou H, Langston C, Beaudet A, 1997).

The contribution of foreign transgene products to the observed immune responses is not unexpected, but it represents a substantial hurdle for gene therapy of hereditary diseases. The immune response to the transgene product may be dependent on the nature of the mutation that affects the endogenous gene. Recently, it has been demonstrated that persistent expression of the foreign antigen (i.e. clotting factor IX in an murine haemophilia B model) may even induce tolerance to the therapeutic antigen (Mingozzi F, Liu YL, Dobrzynski E, et al.2003).

The immunogenicity of the transgene may be depend on the synthesis of the neoantigen in the antigen-presenting cells.(De Geest BR, Van Linthout SA, Collen D,2003) Hence, it will be essential to monitor immune responses to the transgene products in all patients enrolled in gene-therapy studies.

Obviously, strategies to prevent the cellular and humoral immune responses, e.g. adaptive immunity towards the adenovirus vector and to the transgene product may lead to significant improvement of gene therapy of hereditary diseases.

Hence, research is mainly focused on these components of the host's immune response. However, the innate immune responses, as a first line of defence also influence vector persistence. The non-specific innate immune response acts rapidly after viral entry. The early phase of the host's immune response is predominantly brought about by neutrophils, macrophages and natural killer (NK)

cells and lasts about 4 days, until the adaptive immune response is fully activated. (Ginsberg HS, Prince GA. 1994).

The activation of the adaptive immune response is much more rapid upon re-infection of the same pathogen, which is due to the so-called immunologic memory.

After intravenous administration of a first-generation Ad vector to immunocompetent and immunodeficient mice, 90% of the viral genome was eliminated from the liver within the first 24 h. Similar vector elimination was seen after intratracheal administration, although in this case the vector loss was 70% within 24 h Worgall S, Leopold PL,Wolff G, et al 1997). After the fatal incident in a clinical trial for treatment of patients with a deficiency in the liver enzyme ornithine transcarbamylase (OTC) (Raper SE, Yudkoff M, Chirmule N, et al. 2002) , it became evident that the innate immune system is highly activated by intravascular administration of high doses of Ad vectors (Zhang Y, Chirmule N, Gao GP, et al.2001) . In mice, macrophages and dendritic cells mediate an acute cytokine response. Within 6 h high amounts of IL-6, IL-12 and TNF-α are released. So, besides modulation of the adaptive immune response, the modulation of the early immune responses might also be required to facilitate effective gene transfer in vivo with Ad vectors.

In summary, the host response to adenovirus occurs in three phases in animal models and humans (Trapnell, B.C., et al 2002, Pastore, L., et al 1999).

The first phase occurs as early as 1 hour after systemic administration of the virus and continues for 4 days and is characterized by thrombocytopenia, intense periportal polymorphonuclear leukocyte infiltration, and elevated liver enzymes (Raper, S.E., et al 2002; Morral, N et al 2002; Lozier, J.N. et al 2002; Nunes, F.A. et al 2002). These effects are largely due to activation of Kupffer cells which produce and release several cytokines (IL-1, IL-6, TNF-α) and chemokines (MIP-2, MCP-1, IP-10 and RANTES) into the general circulation for the recruitment of inflammatory cells (Muruve, D.A et al 1999; Lieber, A. et al 1997). In addition, macrophages and dendritic cells in the spleen and peritoneum also release significant amounts of chemokines and cytokines into the circulation, contributing to neutrophil-dependent hepatic injury and progression toward the second phase

of the inflammatory process (Zhang, Y., et al 2001; Jooss, K. et al 2003). It is thought that virus capsid proteins alone are responsible for induction of the acute phase of the immune response (Brunetti-Pierri, N et al 2004; Higginbotham, J.N. et al 2002; Schnell, M.A., et. Al 2001).

The second phase of the immune response begins 5-7 days after administration of vector and is directed against newly transduced cells. During this intermediate phase, Ad-specific CD8+ T cells directed against cells expressing viral genes and transgene products are generated (Jooss, K., et al 1998 Morral, N., et al 1997; Yang, Y., et al 1996 Yang, Y. et al 1994). Helper CD4+ T cells, largely stimulated by the presence of adenovirus capsid proteins, are also generated (Kafri, T et al 1998 Yang, Y., 1995). Removal of transduced cells by activated lymphocytes significantly limits the duration of transgene expression in the immunocompetent host and is responsible for inducing a self-limited inflammation in the liver (Christ, M., et al 2000. Worgall, S., et al 1997). At high viral loads, this can progress to severe and lethal liver necrosis, disseminated intravascular coagulopathy, bleeding and, in a few cases, a severe immune response syndrome characterized by multiorgan failure and sepsis (Raper, S.E., et al 2003 Lozier, J.N., et al 1999). These effects have been seen in the clinic and in nonhuman primates but are less pronounced in rodent models used for pre-clinical testing of vectors. The chronic host response continues for several weeks to several months after Ad administration. During this time, cells initially expressing low levels of viral genes and transgene products are recognized and eliminated by cytotoxic T cells (Schowalter, D.B. et al 1999; Tripathy, S.K., et al 1996). CD4+ T-cell-dependent humoral immunity is responsible for the production of anti-adenoviral neutralizing antibodies, which rapidly clear the virus from the circulation and prevent successful gene transfer upon readministration (O'Riordan, C. et al 2002).

4. Strategies for circumvention of vector immunity

In order to improve the clinical application of Ad vectors, it is most important to reduce or evade the vector immune response and enhance target cell transduction. Several approaches have been developed to meet these contradictory requirements for improving the efficacy of Ad vector-based gene transfer.

4.1 Immunosupression or immunomodulation

It has been shown that the use of immunosuppressive agents, such as cyclosporin, cyclophosphamide (Smith et al. 1996), deoxyspergualin (Kaplan et al. 1997), FK506, (Ilan et al. 1997) and CTLA4Ig (Guerette et al. 1996; Jooss et al. 1998), or transient depletion of CD4 lymphocytes using an anti-CD4 monoclonal antibody (Ye et al. 2000), use of anti-CD40 ligand antibody to block CD40-CD40 ligand interactions (Chirmule et al. 2000), and oral tolerization to Ad proteins (Ilan et al. 1998) enhance the duration of transgene expression following systemic delivery of Ad vectors. These approaches help in inhibiting humoral, cell-mediated, or both responses to Ad. Since macrophages play an important role in the induction of innate immune response following vector inoculation, depletion of macrophages and dendritic cells in the liver and spleen following administration of liposome-encapsulated dichloro-methylene-biphosphonate resulted in reduced cytokine production (Zhang et al. 2001). Short-term depletion of hepatic macrophages resulted in increased hepatic transgene expression and reduced transgene-specific humoral immune response following Ad vector inoculation in mice (Schiedner et al. 2003b). Similarly, depletion of alveolar macrophages prior to intratracheal administration of an Ad vector improved vector transduction and persistence in both immunocompetent and immunodeficient mice (Worgall et al. 1997). The use of immunosuppressive agents or depletion of macrophages will not be preferred in clinical cases due to the inherent toxicity of such strategies. Nevertheless, these studies have demonstrated the feasibility of manipulation of the host innate immune response

against Ad vectors to allow increased vector survival and prolonged transgene expression.

4.2 Altering native Ad vector and cell surface receptor interactions

Ad5 attachment to a susceptible cell occurs via the interaction between the Ad fiber knob and cosackievirus adenovirus receptor (CAR) on the host cell membrane (Bergelson et al. 1997; Tomko et al. 1997). CAR is a member of the immunoglobulin superfamily and serves as a high-affinity receptor for Ad in families A, C, D, E, and F but not B. In addition, major histocompatibility (MHC) class I α2 domain (Hong et al. 1997), heparin sulphate glycosaminoglycan (Smith et al. 2003) or sialic acid saccharide (Arnberg et al. 2000) may also serve as the primary receptor for Ad.

In addition to these primary receptors, host cell integrins serve as co-receptors for Ad entry (Wickham et al. 1993). The Ad penton base protein interacts with vitronectin-binding integrins, specifically αvβ3 and αvβ5, for virus uptake (Wickham et al. 1993). This process is facilitated by the arginine-glycine-asparagine (RGD) motif of the penton base. Interestingly, the RGD motif is also found in a number of adhesion molecules that are known to interact with integrins (Bai et al. 1993). The interaction of Ad penton and αvβ1 integrins promotes actin cytoskeletal reorganization via activation of several signalling molecules (Li et al. 2001). Binding of the Ad5 fiber knob to CAR receptor could be effectively prevented with a knob specific antibody. For targeting Ad5 vectors to receptors other than CAR, knob-specific neutralizing antibody could be complexed either to a specific ligand or a receptor-specific antibody (Bilbao et al. 1998) ***(Fig. 7)***. This complex molecule will efficiently bind Ad knob on one side and a specific receptor on the other side. With this technology, a wide variety of HAd5 vectors have been successfully targeted to a number of receptors including folate, epidermal growth factor, fibroblast growth factor, epithelial cell adhesion molecule (EpCAM), tumor-associated glycoprotein (TAG)-67, and CD40 (Bilbao et al. 1998). These modified vectors should be preferentially taken up by the specific cells.

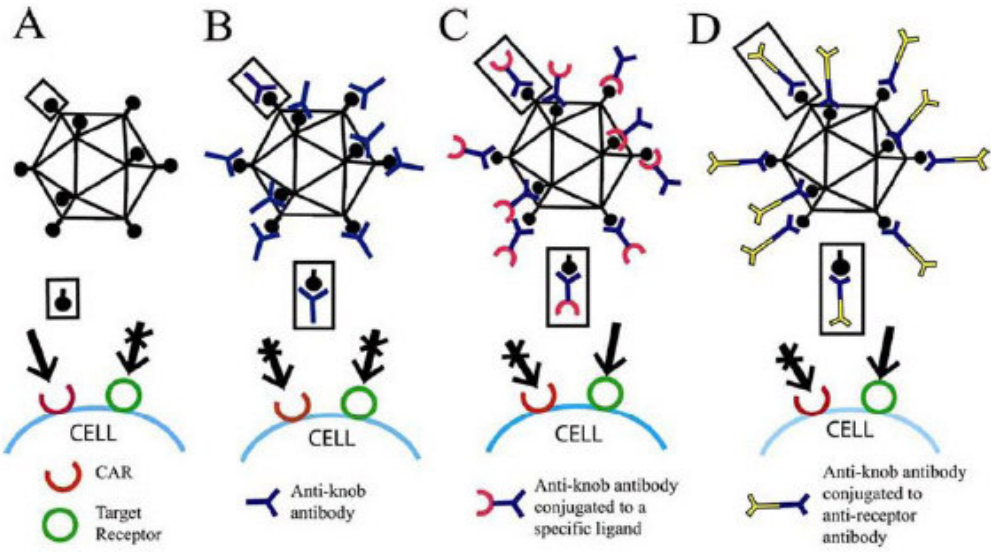


Figure 7 Some of the strategies for designing targeted adenoviral (Ad) vectors: A) Binding of adenovirus to cells via the knob domain of the fiber to CAR. B) Adenovirus complexed with an anti-knob antibody fails to bind to CAR. C) Conjugation of a specific ligand to the anti-knob antibody would allow virus binding to the targeted receptor on the cell surface. D) Conjugation of antireceptor antibody to anti-knob antibody would target the Ad vector to the specific receptor on the cell surface.

These modified vectors should be preferentially taken up by the specific cells. Ad5 fiber knob has been shown to induce dendritic cells activation and maturation (Molinier-Frenkel et al. 2003). Virus-induced maturation of dendritic cells was significantly reduced when knobless Ad particles were incubated with immature dendritic cells. Therefore, fiber knob modifications to incorporate cellular ligands with novel cell-binding capacity might confer targeting and decrease vector immunogenicity. Ad fiber and CAR interactions are considered important for preferential hepatic sequestration of Ad vectors following intravenous delivery. Uptake of Ad vectors by hepatocytes and Kupffer cells lead to an increase in cytokine and chemokine mRNA expression, and subsequently an enhanced innate immune response (Schoggins et al. 2005).

Fiber-pseudotyped Ad vectors were found to induce significantly lower innate immune response following systemic delivery, highlighting the importance of fiber-modification in Ad gene delivery. Similarly, immunogenicity of a chimeric vector containing Ad35 capsid and Ad5 fiber knob was enhanced indicating a potential role of the fiber knob in the immunogenicity of Ad5 vectors (Nanda et al. 2005). It is very important to mention here that, despite a lower innate immune response, adaptive cellular and humoral responses were not affected by fiber modification. Since virus neutralizing antibodies are primarily directed to Ad hexon (Ostapchuk et al. 2001), it is anticipated that modification of hexon will evade vector immunity.

Targeting of Ad vectors could also be achieved by fusing the extracellular domain of CAR to peptide-targeting ligands (Kim et al. 2002). Genetic targeting of Ad vectors by engineering small peptides into the Ad fiber (Aoki et al. 2001), protein IX (Zakhartchouk et al. 2004) or by replacing the fiber protein with the phage T4 fibritin (Krasnykh et al. 2001) has been also demonstrated, but the size of the peptide appears to be a limitation. Similarly, the use of bifunctional polyethylene glycol molecules is useful in ablating the vector tropism by CAR-mediated interaction and providing specific vector targeting by incorporating a ligand for a particular receptor (Lanciotti et al. 2003).

4.3 Vector microencapsulation

The use of polyethylene glycol-cationic lipid to coat Ad vectors (Chillon et al. 1998) and poly (lactic-glycolic) acid (PLGA) copolymer encapsulation (Beer et al. 1998) has also been shown to elude virus-neutralizing antibodies. Sodium alginate-based biodegradable microparticles have been shown to encapsulate purified protein, bacteria, DNA or viruses and can be delivered to the animals by various routes of inoculation (Mittal et al. 2000). Since alginate microspheres are biodegradable and no harsh treatments or organic solvents are used in the process of their synthesis, the viability of Ad vectors in these microparticles is usually very high. Encapsulation of an Ad5 vector into alginate microparticles could effectively evade the vector-specific immune response (Sailaja et al. 2002). More than 70% of alginate microspheres are approximately 5–10 μm in size, and therefore, it is expected that majority of them will be taken up by macrophages and dendritic cells (Lomotan et al. 1997). It appears that alginate microspheres may be an attractive delivery system to target macrophages and dendritic cells, but there is a need to study the role of these microparticles in modulating the immune response through macrophages and dendritic cells.

Use of bilamellar cationic liposomes to encapsulate Ad vectors also provided protection from preexisting humoral immune responses (Yotnda et al. 2002). Similarly, microsphere-liposome complexes gard Ad vectors from neutralizing antibody responses and are capable of effectively transducing cells leading to successful transgene expression (Steel et al. 2004). It seems that transgene expression levels by encapsulated vectors are usually lower (approximately 50–70%) than those of unencapsulated vector both in the naïve and vector primed animals (Sailaja et al. 2002). It may be due to slow release of the vector from microparticles over time that will also prolong the duration of transgene expression.

4.4 Use of alternate had serotypes (serotype switching)

Since more than 50 Ad serotypes exist, and the neutralizing humoral immune response to Ad is serotype-specific, another strategy to overcome Ad vector immune response could be serotype switching in vector construction (Parks et al. 1999). Subgroup B Ad, such as Ad3, Ad11, and Ad35, have been shown to utilize the membrane cofactor protein CD46 as an attachment receptor
(Gaggar et al. 2003; Segerman et al. 2003; Sirena et al. 2004). This particular feature makes these viruses attractive for targeting cell types that are refractory to HAd5 vectors that are primarily dependent on CAR-mediated internalization. Low seroprevalence of Ad11 and Ad35 makes them promising vectors for in vivo applications. Replication-defective Ad35 vectors efficiently transduced human cells and eluded preexisting Ad immunity (Gao et al. 2003). Similarly, Ad11 based replication-defective vectors have shown expanded tropism. Ad35 based replication-defective vector vaccines evaded preexisting Ad5 immunity in mice (Barouch et al. 2004) as well as in rhesus monkeys (Shiver et al. 2004).

4.5 Use of Helper-Dependent Ad (HD-Ad) Vectors

HD-Ad vectors are constructed by removing all coding sequences of the Ad genome except the packaging sequence and inverted terminal repeats, thereby eliminating the problem of residual viral gene expression associated with E1/E3-deleted Ad vectors (Mitani et al. 1995; Parks et al. 1996). Initial studies showed that HD-Ad vectors elicited limited cell-mediated immune response, had high cloning capacity, and produced long-term gene expression in both naïve small laboratory animals (Morsy et al. 1998; Schiedner et al. 1998), and nonhuman primates (Morral et al.1999) without causing significant liver damage and toxicity. Systemic delivery of HD-Ad vectors has been shown to provide strong transgene expression for prolonged period with minimal toxicity in the baboon, mouse, or canine model (Brown et al. 2004; Morral et al. 1999).

HD-Ad vectors also induce vector-specific immune response similar to that generated by E1-deleted Ad (Roth et al. 2002). Systemic administration of HD-Ad vectors in baboons also leads to acute toxicity accompanied by activation of the innate response in a dose-dependent manner (Brunetti-Pierri et al. 2004) indicating that vector-mediated acute toxicity is independent of viral gene expression. Sequential delivery of different HD-Ad vector serotypes circumvented the humoral response to the virus (Morral et al. 1999) suggesting that long-term transgene expression was possible by sequential delivery of HD-Ad vectors of different serotypes. However, acute toxicity due to vector is not prevented or reduced, implying the importance of the viral capsid components in vector toxicity.

4.6 Use of Nonhuman Ad Vectors

Since Ad viruses are species-specific, nonhuman Ad are expected to be no prevalent in humans, and therefore, they evade preexisting Ad immunity. In order to extend the range of Ad vectors that could be used to evade Ad neutralizing immune response, a number of nonhuman Ad such as bovine Ad type 3 (BAd3) (van Olphen et al. 2002), canine Ad type 2 (Hemminki et al. 2003), ovine Ad (Hofmann et al. 1999), chimpanzee Ad (Farina et al. 2001), and porcine Ad type 3 (Bangari et al. 2004) were exploited for vector construction. It has been shown that nonhuman Ad vectors infect human cells in culture leading to expression of the transgene (Bangari et al. 2004; Bangari et al. 2005). Since HAd5-, BAd3- and PAd3-specific neutralizing antibodies do not cross-neutralize (Moffatt et al. 2000), it is expected that sequential administration of Ad5, BAd3 and PAd3 would effectively evade the vector specific neutralizing immune response. The sera of mice immunized with Ad serotypes 2, 4, 5, 7, and 12 did not neutralize chimpanzee Ad (Farina et al. 2001) indicating the utility of such vectors in evading Ad preexisting immunity. Following the decline in transgene expression to background levels, readministration of the vector is necessary to maintain therapeutic levels of transgene expression; it seems that sequential administration of nonhuman Ad vectors could provide that opportunity.

5. PEGylation of adenoviral vectors

Another approach to circumvent both cellular and humoral immune responses generated against Ad is biochemical modification of viral capsid proteins with non-toxic materials to shield the virus from the immune system. Covalent modification of biological molecules with functionalized poly(ethylene glycol) (PEG) has been studied since the late 1970s. Poly (ethylene) glycol has a low toxicity profile, does not have immunogenic properties and has been approved by the Federal Food and Drug Administration (FDA) for use in foods, cosmetics and pharmaceuticals including parenteral, topical, rectal and nasal formulations (Working, P.K., et al 1997).

Several PEGylated products have received FDA approval within the last decade: adenosine deaminase (Adagen, Enzon), L-asparaginase (Oncaspar, Enzon), staphylokinase (PEG-SAK, Thrombogenics), interleukin α-2a (PEGASYS, Roche), interleukin α-2b (PEG-INTRON, Schering-Plough) and hemoglobin (Hemospan, Sangart). In most cases, these modified proteins have improved therapeutic efficacy with enhanced circulation half-life *in vivo*, enhanced solubility, and improved *in vivo* bioactivity. This technology has specifically been employed to protect therapeutic proteins and enzymes from neutralization in patients that must receive them as a chronic therapy (Scott, M.D., Murad, K.L., 1998).

Several groups have shown that administration of several doses of a PEGylated antigen induces a state of tolerance against the native antigen in immunocompetent animals. Ito et al. found that animals dosed with a series of six doses of PEGylated hen egg lysozyme (HEL) and rechallenged with six additional doses of unmodified HEL failed to produce detectable levels of anti-HEL IgG antibodies (Ito, H.O., 1998). So et al. also found that immunization of animals with PEGylated HEL effectively blunted the T cell mediated immune response against the native antigen (So, T., et al 1999). Further studies suggest that the extent by which the molecule is covered by PEG dictates the level of immunological tolerance to the antigen. Others have shown that increasing the molecular weight and branching of the functionalized PEG molecules also

effectively blunts the immune response against the native antigen in some instances (Caliceti, P., et al 1999). Mechanistic studies by Sehon and others suggest that the immunosuppression induced by PEG-antigen conjugates can be attributed to the production of antigen-specific suppressor T cells and by suppressor factors produced by these cells which in turn reduce the production of antigen-specific T and B cells (Sehon, A.H., Carl Prausnitz, 1991).

5.1 Preliminary studies

A method for the rapid conjugation of functionalized monomethoxypoly(ethylene) glycol (MPEG) activated by either tresyl chloride (TMPEG), succinimidyl succinate (SSPEG) or cyanuric chloride (CCPEG) to free lysine groups on the capsid proteins of E1/E3 deleted recombinant Ad vectors has been established in Maria Croyle laboratory . This process is simple to perform, is complete within 2-3 hours and does not compromise the transduction efficiency of the virus ***(Fig. 8)***. This effect is probably due to the new physical properties of the PEGylated virus. PEGylation significantly reduced the negative surface charge of the virus capsid, which could promote non-specific interaction with the cell surface (Croyle, M.A., et al 2000). This modification also increased the partition coefficient of the virus, which would allow it to indiscreetly partition though cell membranes. While enhanced transduction efficiency could be an added benefit of PEGylation, it raises some additional issues about these new vectors. Conjugation of high molecular weight PEGs to various biomolecules can extend residence time in the circulation from minutes to days. This effect, in combination with a general increase in transduction efficiency could promote efficient transduction of tissue for which the vector was not intended. It is also unclear how enhanced circulation times of adenoviral vectors will affect the overall health of the patient.

It has been also found that humoral and cellular immune responses were reduced in immunocompetent animals after a single intravenous dose of the modified vectors ***(Fig. 9)***. Th1 (IFN-γ and IL-2) and Th2 (IL-10) responses were observed in splenocytes from mice that received the native virus as evidenced by the stimulated production of subtype specific cytokine. Th1 responses were significantly reduced in samples from animals given PEGylated viruses.

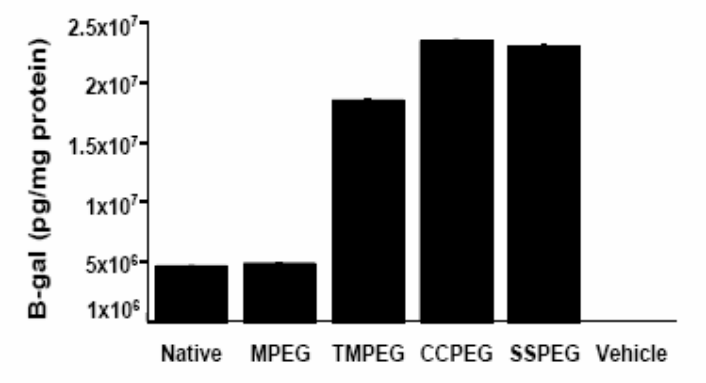


Figure 8 Transduction Efficiency of PEGylated Ad in Mouse Liver: C57BL/6 mice were injected intravenously ($5x10^{11}$ vp/kg) with either unmodified (native) or PEGylated Ad encoded with the betagalactosidase transgene. Four days after injection, animals were necropsied and gene expression assessed in liver homogenates by a beta-galactosidase ELISA. Data reflect the average values from 10 mice/treatment group. Error bars reflect the standard error of the mean of the data. MPEG – virus formulated with inactivated PEG included in the conjugation reactions but which is not linked to capsid proteins.

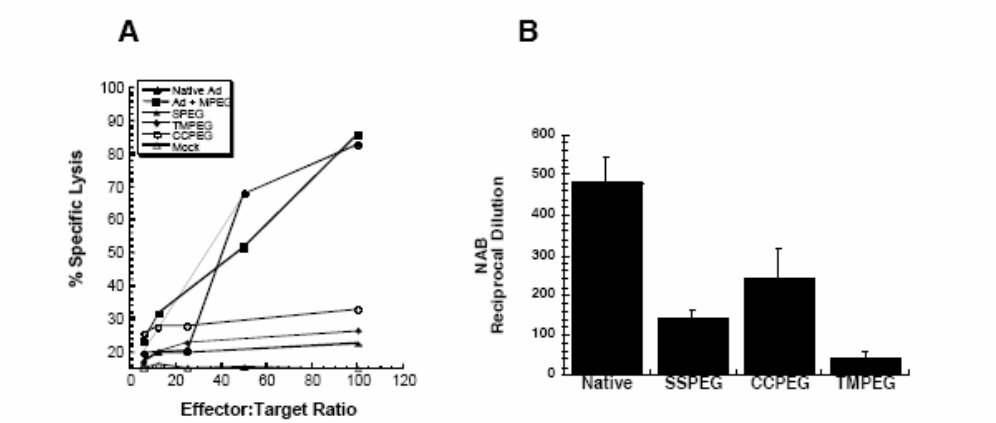


Figure 9 PEGylation of adenoviral capsid proteins reduces the cellular and humoral response against the virus: A) Spleen cells were obtained from mice 10 days after intravenous administration of PEGylated virus, unmodified adenovirus (native Ad) or unmodified virus formulated with 0.1% unactivated PEG (MPEG) at a dose of $5x10^{11}$ vp/kg particles/kg and assessed for adenovirus-specific CTL responses when cultured with targets infected with the native virus by a chromium release assay. B) Anti-adenovirus neutralizing antibody levels measured in serum of mice 30 days after administration. Data are the average results from ten animals per treatment group studied in two separate experiments.

SSPEG and TMPEG preparations stimulated Th2 type responses. Splenocytes from animals given these vectors produced IL-10 levels twice that of animals given unmodified virus. In each analysis, samples from animals that received a dose of unmodified virus in a formulation of PBS and 1% unactivated poly(ethylene) glycol (MPEG) generated responses similar to that seen with unmodified virus, indicating that PEG alone does not effect the immune response generated against adenoviral vectors independent of its modification of capsid.

Mitigation of the immune response was the driving force for the development of PEGylated Ad vectors. After reviewing their immunological and physical stability profiles, vectors modified with the SSPEG chemistry were selected for additional studies to determine how this modification affected the acute phase of the innate immune response. In this study, C57BL/6 mice were given a single intravenous dose of 4 x 10^{12} particles/kg of either an unmodified E1/E3 deleted Ad expressing beta-galactosidase, an unmodified HD-Ad expressing the same transgene or PEGylated versions of each vector. Similar levels of transgene expression were found in all treatment groups. Despite this fact, PEGylation significantly reduced IL-6 levels in animals treated with the E1/E3 deleted vector by a factor of 4 and with the HD vector by a factor of 2 ***(Fig. 10)***. IL-12 production was reduced by a factor of 3 in animals treated with the modified E1/E3 deleted virus (Figure 10B). A similar drop was detected in animals treated with PEGylated HD Ad. PEGylation of each vector reduced TNF-α levels to baseline levels (Figure 10C).

Other biochemical markers suggest that PEGylation may adequately dampen the innate response in a manner that reduces vector-induced tissue damage, coagulopathy and inflammation. It has been measure serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels as markers of tissue and liver damage and platelet counts as a marker for coagulopathy associated with vector administration. Even though each vector produced similar levels of gene expression, elevated serum transaminases with respect to saline controls were only detected in animals treated with the unmodified (native) E1/E3 deleted Ad *(Table 2).* AST and ALT levels of animals treated with the PEGylated vectors fell within the normal range for C57BL/6 mice (Quimby, F.W., 1999).

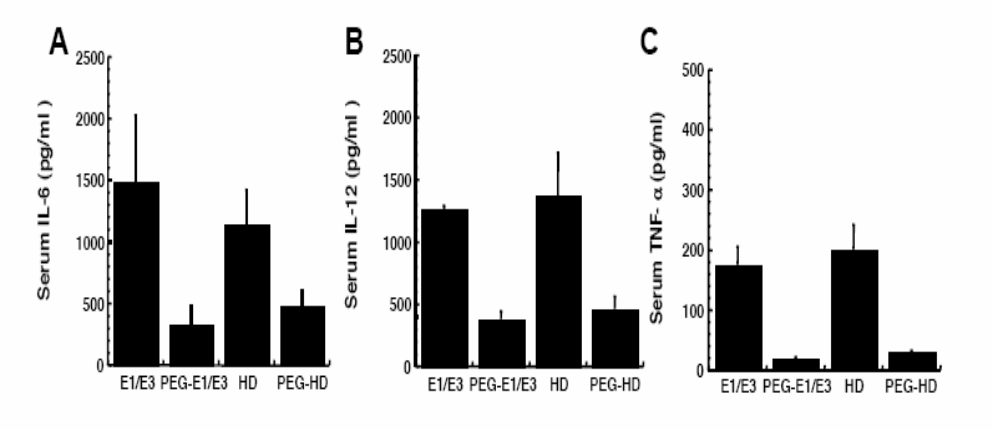


Figure 10 PEGylation of recombinant Ad reduces serum levels of pro-inflammatory cytokines: Serum levels of IL- 6 (A), IL-12 (B) and TNF-alpha (C) were assessed from serum samples taken from C57BL/6 mice 6 hours after systemic administration of a single dose of $4x10^{12}$ particles/kg of either unmodified E1/E3 deleted Ad expressing the beta-galactosidase transgene (E1/E3), unmodified helper-dependent adenovirus Ad-SRα-βGEO (HD), or the PEGylated versions of each virus (PEGE1/ E3, PEG-HD). Data reflect average values + the standard error of the mean for five mice from each treatment group.

	Treatment Group			
Dose	E1/E3 Adenovirus		HD Adenovirus	
Day 3	ALT (U/L)	AST (U/L)	ALT (U/L)	AST (U/L)
Native	601 ± 96.2**	405 ± 68.8**	39.3 ± 5.4	205.7 ± 44.3
PEG	46.7 ± 16.0	228.7 ± 15.7	35.5 ± 5.0	254 ± 37.9
Saline Controls	31.2 ± 3.4	212.2 ± 19.5		
Day 7				
Native	472 ± 148.4*	506.7 ± 68.5**	53.7 ± 15.1	125 ± 18.03
PEG	50.7 ± 20.3	280.3 ± 29.8	42 ± 5.4	171.3 ± 11.6
Saline Controls	54 ± 8	221 ± 10.3		
Normal Range[a]	24-140	72-288		

Table 2 Serum transaminase levels of mice after systemic administration of unmodified and PEGylated Adenoviruses: Saline controls were bled 3 and 7 days after treatment in the same manner as animals dosed with virus. Data represent the mean + standard error of 4 mice per treatment group. AST, aspartate transaminase; ALT, alanine transaminase; U/L, units per liter. $^{**}p = 0.001$, $^{*}p = 0.01$.

Three days after vector administration, a significant drop in platelet count from was detected in animals treated with unmodified HD-Ad, which resolved to baseline levels 4 days later ***(Fig. 11)***. Animals treated with the PEGylated vector did not experience a drop in platelet count 3 days after vector administration. Platelet counts from this group were not statistically different than those from animals treated with a saline bolus and remained unchanged throughout the remainder of the study. Results were similar for the E1/E3 deleted vectors.

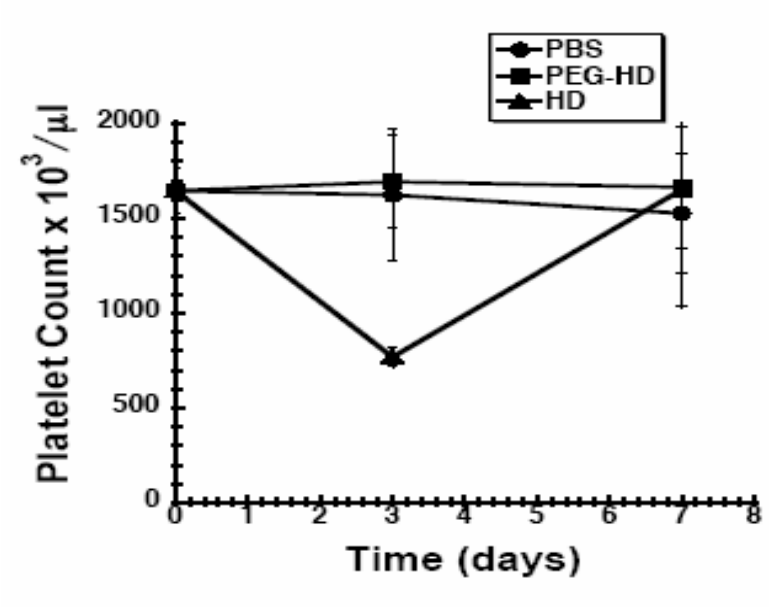


Figure 11 PEGylation of helper-dependent adenovirus prevents vector-induced thrombocytopenia after systemic delivery: Fourteen days prior to vector administration, baseline platelet counts were determined (t=0). Mice were dosed with either $8x10^{10}$ viral particles of unmodified (HD) or PEGylated (PEG-HD) Ad-SRα β-GEO or 100 microliters of saline (PBS, vehicle control). At each timepoint, mice were terminally bled and half the blood collected in tubes treated with sodium citrate for platelet analysis. Data reflect average values + the standard error of the mean for five mice from each treatment group.

CHAPTER II

MATERIALS AND METHODS

1. HDΔ28E4LacZ production

1.1 Helper virus

The helper virus (HV) using in this work, AdNG163R-2, is a first generation adenoviral vector that contains a reverse orientation ψ (with respect to the HDAd) flanked by loxP sites so that it is excised in Cre-expressing cells to render the HV genome unpackageable (Donna Palmer and Philip Ng, 2003) . Therefore, AdNG163R-2 contains a DNA stuffer, composed of noncontiguous λ DNA segments with no intact coding sequences, into the wild-type E3 region; the HV have a large genome size (37.2 kb) so that maximum separation from HDAds (designed to be ~29 kb) can be achieved if necessary because a common and successful strategy to minimize HV contamination is physical separation from the HDAd by CsCl ultracentrifugation based on differences in their genome sizes (Sakhuja, K., et al. 2003).

1.2 Producer cell line 116

Cre-expressing producer cells (116) derive from 293N3S cells (Graham, F. L. 1987), a 293 derivative capable of both adherent and suspension growth. 116 were generated by transfecting 293N3S cells with pNG159, a plasmid that contains the Cre-ricombinase and hygromycin-resistance coding sequences, separated by the encephalomyocarditis virus internal ribosome entry site.

Monolayer of 116 cells was grown in MEM (Life Technologies) supplemented with 10% FBS (Invitrogen) and 50 μg/ml hygromycin (Sigma). Joklik's modified MEM (Biowittaker) supplemented with 10% FBS and 50 μg/ml hygromycin was

used to grow 116 cells in suspension. Suspension 116 cells were grown in spinner flasks (Bellco) at 37°C on a magnetic stir plate.

1.3 HDΔ28E4LacZ rescue, amplification, and large-scale production

HDΔ28E4LacZ was rescued (serial passage P0) by transfecting a 60-mm dish of 116 cells at ~80% confluency with 20 µg of *Pme*I-digested pΔ28E4LacZ followed, the next day, by infection with 1000 vp/cell of AdNG163R-2. HDΔ28E4LacZ was amplified by serial coinfections of 60-mm dishes of 116 cells at ~90% confluency with 10% of the crude lysate from the previous passage and 200 vp\cell of AdNG163R-2 for serial passages 1, 2, 3 (P1, P2, P3). For P4, two 150-mm dish of 116 cells at ~ 90% confluency were coinfected with 10% of the crude serial passage 3 lysate and 200 vp/ cell of AdNG163R-2. All serial passages were harvested 48 h postcoinfection for use as inoculum for the subsequent passage after one freeze–thaw. 24 h after the infection every amplification passage was controlled by 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) staining.

Large-scale HDAd production was performed as follows: 3 liters of 116 cells at 3–4 x 10^5 cells/ml was harvested by centrifugation and resuspended in 5% volume conditioned medium and coinfected with 100% of the crude lysate from the 150-mm dish of serial passage 4 and 200 vp/cell of AdNG163R-2. Virus adsorption was performed at 37°C on a magnetic stir plate for 2 h, after which medium (25% conditioned and 75% fresh Joklik's MEM supplemented with 5% FBS and 25 mM HEPES) was added to a final volume of 2 liters. Coinfected cells were harvested 48 h later for lysis and resuspended in TM solution (Tris HCl pH 8 1M and $MgCl_2$ 1M)

Rescue, amplification, and large-scale production of HDAd are summarized in ***figure 12***.

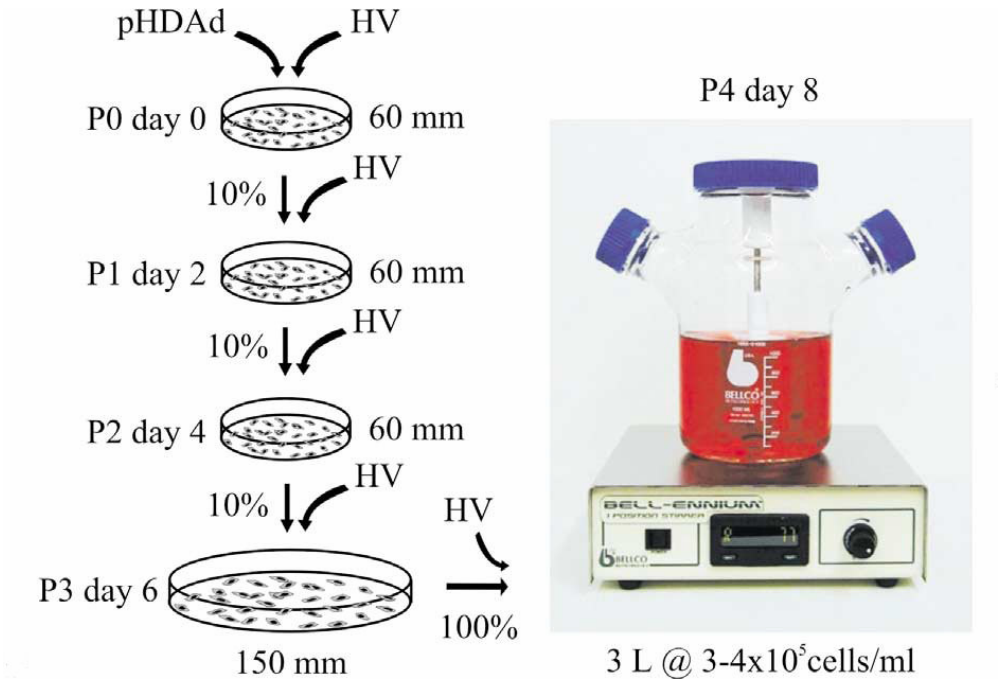


Figure 12 HDAd rescue, amplification, and large-scale production: Overview of the rescue (P0), amplification (P1 to P4), and large-scale production (P5) of HDAd. The entire process beginning with vector rescue (P0) to the final purified HDAd takes less than 2 weeks.

1.4 HDΔ28E4LacZ purification

The harvested cells were lised by three freeze-thaw cycles and were incubated with $MgCl_2$ 2M and DNase for 1 h at 37°C. After incubation the cellular debris was spinned down and the lysate was subjected to ultracentrifugation on a continuous CsCl gradient with a Beckman SW41 rotor (35,000 rpm for 2 hr at 4°C). The upper band, which contains virions, was eliminated and a second ultracentrifugation on a continuous CsCl gradient (35,000 rpm for 18 hr at 4°C) was performed. The harvested vector was dialyzed in a TM (Tris HCl pH 8 1M and $MgCl_2$ 1M) and was stored at -80° C. Vector concentration (particle number) was determined by UV spectrophotometric analysis at 260 nm: OD_{260} x 50 x 10^{12} vp\ml.

1.5 Southern analysis for helper virus contamination of HDΔ28E4LacZ

To analyze viral DNA for helper contamination, a fraction of 50 µl was incubated with 0.5 mg of proteinase K per ml in 10 mM Tris HCl, pH 7.5/10 mM EDTA/0.5% SDS at 37° C overnight. DNA was prepared by phenol/chloroform extraction and ethanol precipitation. The vector HDΔ28E4LacZ, the helper virus AdNG163R-2 and the plasmid pΔ28E4LacZ were digested with *PstI* and *PmeI* and fractionated on a 1,2 % agarose gel and transferred to a nylon membrane (GeneScreen- Plus; NEN) using a slot blot apparatus (Minifold II; Schleicher). The membrane was hybridized using a probe designed on the ITRs of pΔ28E4LacZ and prepared by PCR (*Polymerase chain reaction*) at this condition: *Initialization step*: 94°C for 10 minutes; *Denaturation step :*94°C for 30 seconds ; *Annealing step*: 58° for 30 seconds; *Extension/elongation step*: 72° for 30 seconds; *Final elongation*: 72° for 3 minutes; *Final hold*: 4°C for indefinite time. The hybridization temperature was of 60°C and the hybridization buffer composed of 5x SSC, 0, 1% SDS, 5% dextran sulphate and 100 µg/ml salmon sperm. The washing were performed at 55°C with a solution of 0, 2% SSC and 0,

1% SDS. The autoradiography was performed with ECL Plus system (Amersham).

1.6 HDΔ28E4LacZ PEGylation

Aliquots of virus were desalted on Econo-Pac 10DG disposable chromatography columns (Bio-Rad, Hercules, CA) and equilibrated with the respective buffer for optimal conjugation. Viral concentrations were determined by UV spectrophotometric analysis at 260 nm. Transduction titer (LacZ-forming units or LFU) was determined by limiting dilution infections of 116 cells. The protein content of viral preparation was determined by a microplate assay with Bio-Rad DC Protein assay reagents and bovine serum albumin as a standard (Bio-Rad). In total, 10 mg of monomethoxypoly (ethylene) glycol activated by succinimidyl succinate (SSPEG, mw 5000, Sigma-Aldrich) was added for each microgram of protein present in the preparation. Conjugation reactions were performed at 25° C with gentle agitation. Reactions were stopped by addition of a 10-fold excess of L-lysine (Sigma-Aldrich) with respect to the amount of PEG added. Unreacted PEG, excess lysine, and reaction by products were eliminated by buffer exchange over a second Econo-Pac 10DG disposable chromatography column (Bio-Rad) equilibrated with 100 mM potassium phosphate-buffered saline (pH 7.4). A separate aliquot of virus was treated and processed in the absence of SSPEG in the same manner as the conjugated virus and served as the unPEGylated control.

1.7 Characterization of PEGylated HD-Ad vectors: capillary zone electrophoresis

PEGylated and unPEGylated adenoviral vectors were characterized by capillary zone electrophoresis using a Beckman PACE 5000 system (Beckman Coulter) with an untreated 50 mm (ID) x 27 cm capillary. The temperature of the capillary was controlled at 221C. A preliminary 2 min wash in 1 N NaOH followed by a 2-

min wash in nanopure water and a third wash in running buffer (20 mM sodium phosphate, pH 7.0, 5 mM sodium chloride) were performed prior to sample analysis. A voltage of 17 kV was used for the separations and the detector signal at 214 nm was recorded and processed by the PACE station software package.

2. Animal studies

2.1 Biochemical analysis

Four male baboons (*Papioi* sp., 6-7 kg) had a 22-gauge catheter placed in the left cephalic vein for sample collection and administration of virus. Blood samples were taken prior to vector administration for blood cell counts (PT, aPTT, platelets, WBC, neutrophilis) and blood chemistry analysis (ALT, LDH) to establish baseline levels. Animals received one of two doses ($5x10^{11}$vp/kg and $3x10^{12}$ vp/kg) of either an unmodified or PEGylated HD-Ad expressing beta-galactosidase. Animals were sedated with ketamine (10 mg/ml) prior to vector administration. Viruses were infused over 2-3 minutes and were followed by flushing the line with sterile saline.

Blood samples (7 ml) were taken at various time points (1, 3, 6, 24, 48, 72 and 96 hours). Two milliliters were used for cytokine analysis and DNA extraction. Cytokine levels (IL-6, IL-12, TNF-α) were determined with commercially available ELISA kits (Biosource International, Camarillo, CA). Viral DNA was isolated from tissues using DNeasy Tissue kits (Qiagen) according to the manufacture's protocol and DNA concentration and purity were determined spectrophotometrically. 4 milliliters were used to determine blood cell counts (PT, aPTT, platelets, WBC, neutrophilis) and blood chemistries. Urine was collected at each time point via a catheter inserted in the bladder and feces will be collected daily for DNA extraction to determine the amount and how long the virus is shed from treated animals. All chemical parameters were run by the folks in San Antonio.

2.2 Neutralization assay

Antiadenovirus antibody titers of baboons immunized with unmodified HD-Ad were determined by incubating serum samples at 56° C for 30 min to inactivate complement followed by dilution in DMEM in two-fold increments starting from a 1:20. Each dilution (100 ml) was mixed with an aliquot of a standard stock of unmodified adenovirus expressing E. coli beta-galactosidase, incubated for 1 h at 37°C, and applied to HeLa cells in 96-well plates ($2x10^4$ cells/well). In total, 100 ml of DMEM supplemented with 20% FBS was then added to each well. Cells were incubated at 37°C for 24 h. Neutralizing antibody titers were calculated as the highest dilution at which 50% of the cells stained blue by visual inspection.

2.3 X-gal histochemistry

These animals were necropsied 96 hr after vector administration and the histochemistry was done by cryosectioning tissue in OCT and X-gal staining. The tissues were incubate for 3 hours at 37^oC and then for 24 hours at room temperature. After the washing the sections were controstained using Nuclear Fast Red.

3. Real- Time PCR

DNA was isolated from tissues using DNeasy Tissue kits (Qiagen) according to the protocol manufacture's DNA was purified by minicolumns and quantified by spectroscopy. Adenoviral DNA content was determined using spectroscopy. Real-time PCR was performed on the DNA in an Applied Biosystems Prism 7900HT sequence detection system with SDS 2.1 software. The parameters used were 95° C for 10 min, 95° for 10 seconds (45 cycles), 60° for 7 seconds (45 cycles) and 72° for 20 seconds (45 cycles). Reactions were performed in triplicate with no template as controls. Amplifications were carried out in a total volume of 20 µl with SYBR Green PCR Master Mix (Applied Biosystems), 2 µl of DNA template, 4 mM $MgCl_2$, and 5 µM primers HD-Ad specific designed on packaging signal. Serial dilutions of plasmid pΔ28E4LacZ were used as control.

CHAPTER III

RESULTS

1. HDΔ28E4LacZ production in 116 cell line

The HDAd used in this study, HDΔ28E4LacZ, contains an MCMV-LacZ expression cassette (Palmer and Ng, 2003). Four preps of virus were produced in 116 cell line that unlike 293Cre4 cells and other producer cells can also grow in suspension, rendering large-scale HDAd production significantly more efficient and simpler, as well as less costly and labor intensive. We coinfected 2 liters (for prep) of passage 45 116 cells at 3–4 x 10^5 cells/ml with AdNG163R-2 and the crude serial passage 4 lysate from two 150-mm dish . 24 h after the infection every amplification passage was monitored by X-Gal staining ***(Fig. 13)***. Forty-eight hours postcoinfection, we purified HDΔ28E4LacZ by one step and two continuous CsCl gradients ***(Fig. 14)***. Spectrophotometric analysis revealed that we obtained a for each total yield of ~1,5 x 10^{13} vp/ml corresponding to a specific yield of 12,650 vp/cell and a titer of 9 x 10^{11} blue forming units/ml (BFU) as determined by X-gal staining following titration on 293 cells. To analyze HDΔ28E4LacZ in detail, we extracted virion DNA from the step and two continuous CsCl gradients and digested it with *ApaLI* and *PmeI*. Agarose gel electrophoresis analysis revealed that the genomic structure of HDΔ28E4LacZ was indistinguishable from that of the parental pΔ28E4LacZ plasmid, except for the expected absence of the 2.5-kb *Apa*LI–*Pme*I fragment bearing the bacterial plasmid sequences present in pD28E4LacZ, suggesting the absence of vector DNA rearrangements ***(Fig. 15)*** .

To determine the level of HV contamination, we performed Southern analysis with probe ITR to compare the intensities HDΔ28E4LacZ band and an AdNG163R-2 band ***(Fig. 16)***. The results revealed the AdNG163R-2-specific band was undetectable in the virion DNA extracted from all CsCl gradients.

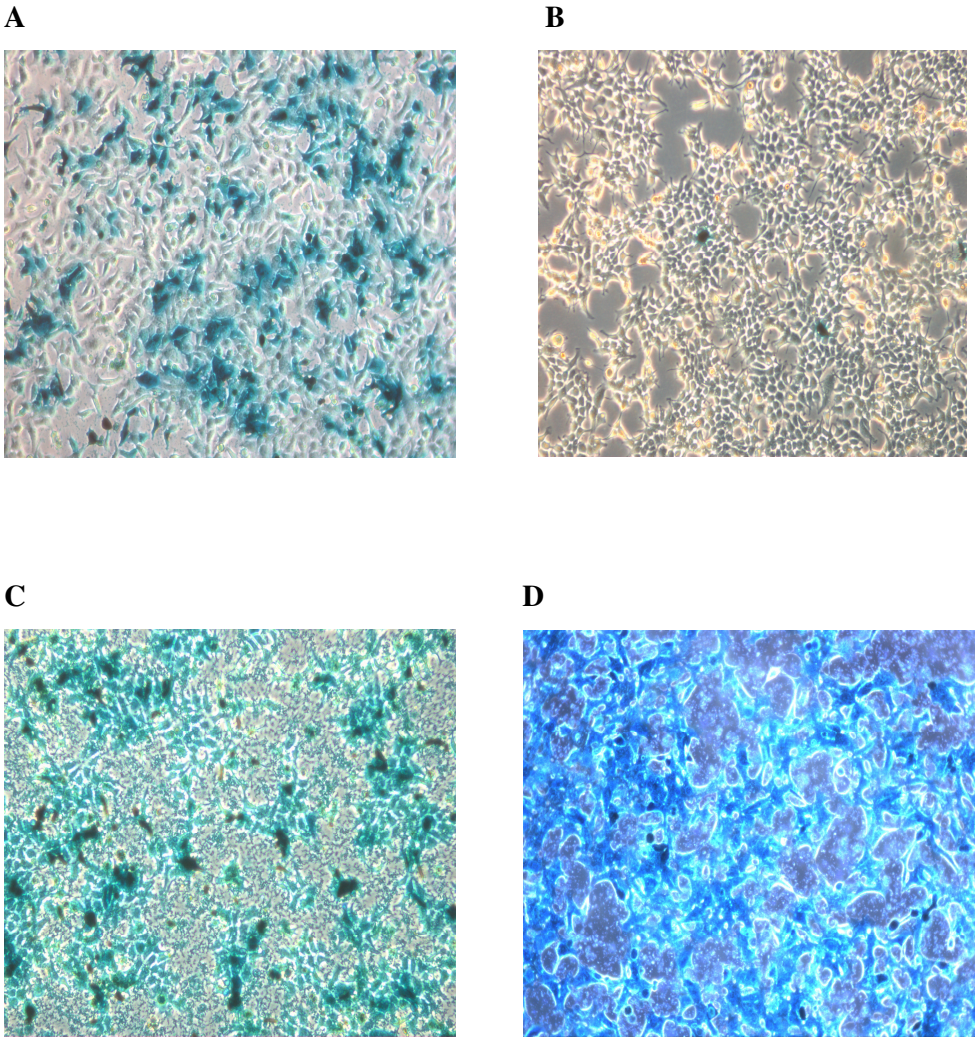


Figure 13 X-gal staining: 24 hours after each amplification passage, the vector amplification was monitored by X-gal staining until to all the cells were stained: this passage was used to infect two 150 mm dishes of 116. (A: P0; B:P1; C:P2: D:P3).

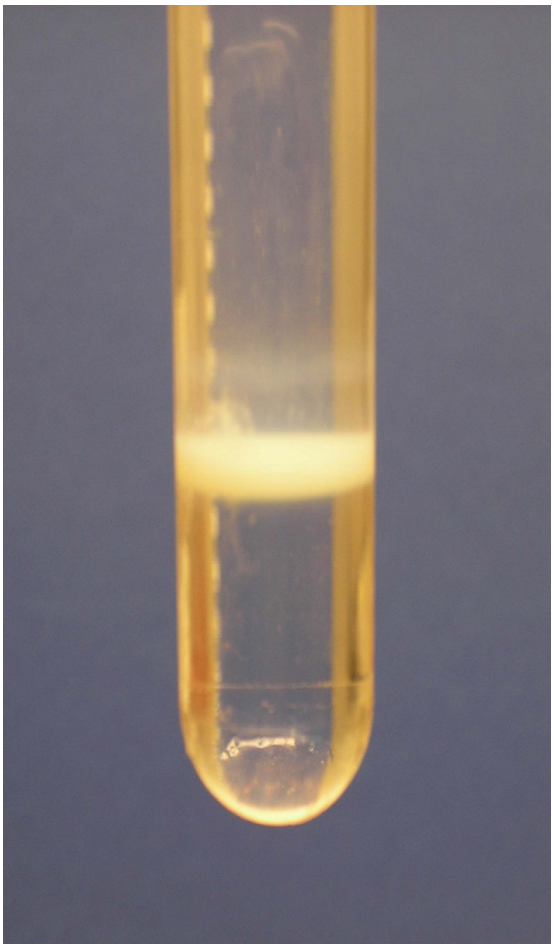


Figure14 HD-Ad purification on cesium chloride: An examples of HD28E4LacZ obtained from 3 liters of 116 cell using AdNG163R-2 as helper virus. The second continuous CsCl gradient is shown.

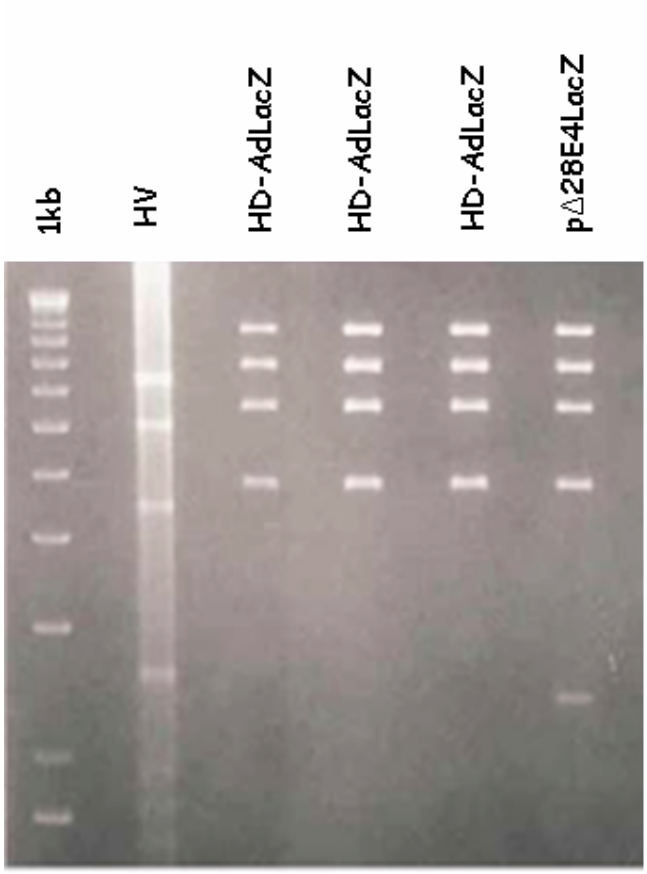


Figure 15 Analysis of HD-AdLacZ DNA: DNA was extracted from the virions obtained from the two continuous CsCl gradients and digested with *Apa*LI. The structure of HDAdLacZ DNA is indistinguishable from that of the parental pΔ28E4LacZ plasmid, except for the expected absence of the 2.5-kb *Apa*LI–*Pme*I fragment bearing the bacterial plasmid sequences present in pΔ28E4LacZ.

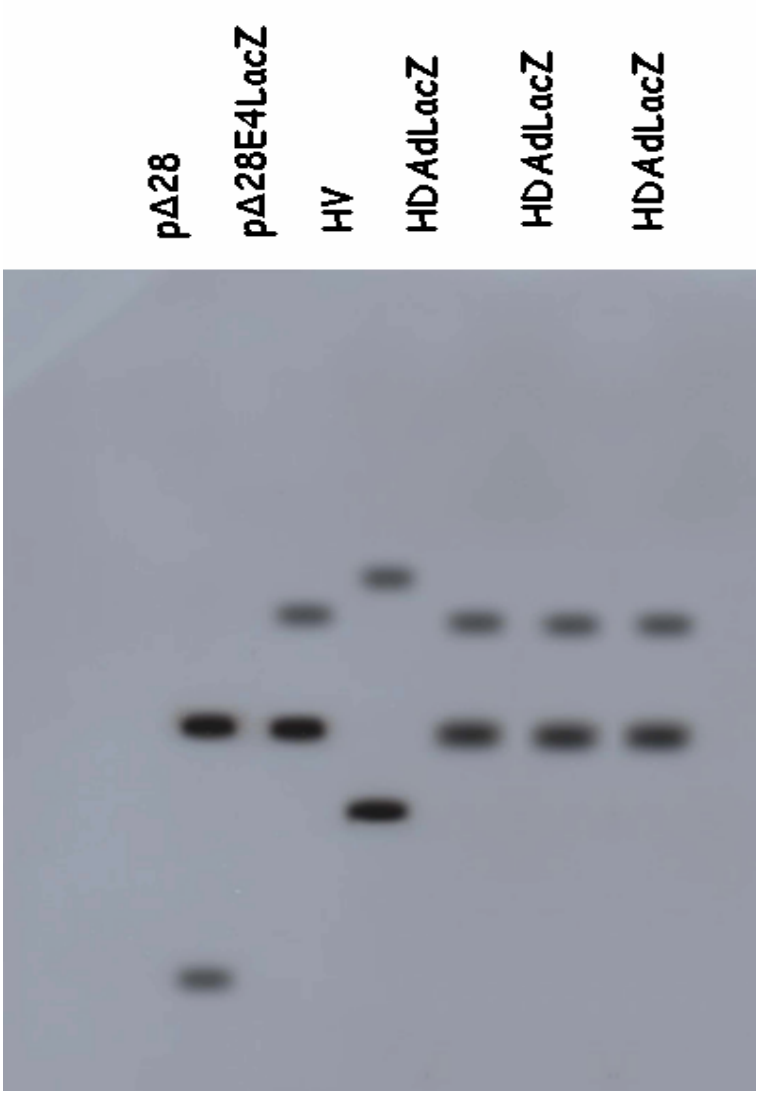


Figure 16 Southern analyses to verify HV contamination: The vector HDΔ28E4LacZ, the helper virus AdNG163R-2 and the plasmid pΔ28E4LacZ were digested with *PstI* and *PmeI.* Using an ITR probe, the AdNG163R-2-specific band was undetectable in the virion DNA extracted from all CsCl gradients.

1.1HDΔ28E4LacZ PEGylation

The succinimidyl propionate group of the SPA-PEG molecule reacts with nucleophilic groups (mostly ε-amino groups of lysine) on proteins to form stable amide bonds. The rate of reaction depends on the concentration of PEG, as well as the length of reaction time, temperature, and pH. In Based upon previous experience with PEGylation of recombinant viral vectors, a 2 h reaction time was selected. Four separate lots of HD-Ad. The average titer of all HD-Ad prepaearations prior to PEGylation was 9×10^{11} BFU/ml. PEGylated preparations had an average titer comparable with titer of preparation preparations prior to PEGylation. The PEGylation reaction was monitored by capillary electrophoresis. Unmodified HD-Ad had a peak at 7.3 min (Peak C), and a similar peak (Peak D) had HD-Ad after the conjugation reaction without PEG. The PEG-HD-Ad vector was detected as a single peak at 6.4 min (Peak B). A standard solution of free PEG produced a single peak at 4.8 min (Peak A) and was absent from the PEGylated preparation, indicating that free PEG was efficiently removed after the conjugation reaction ***(Fig. 17)***.

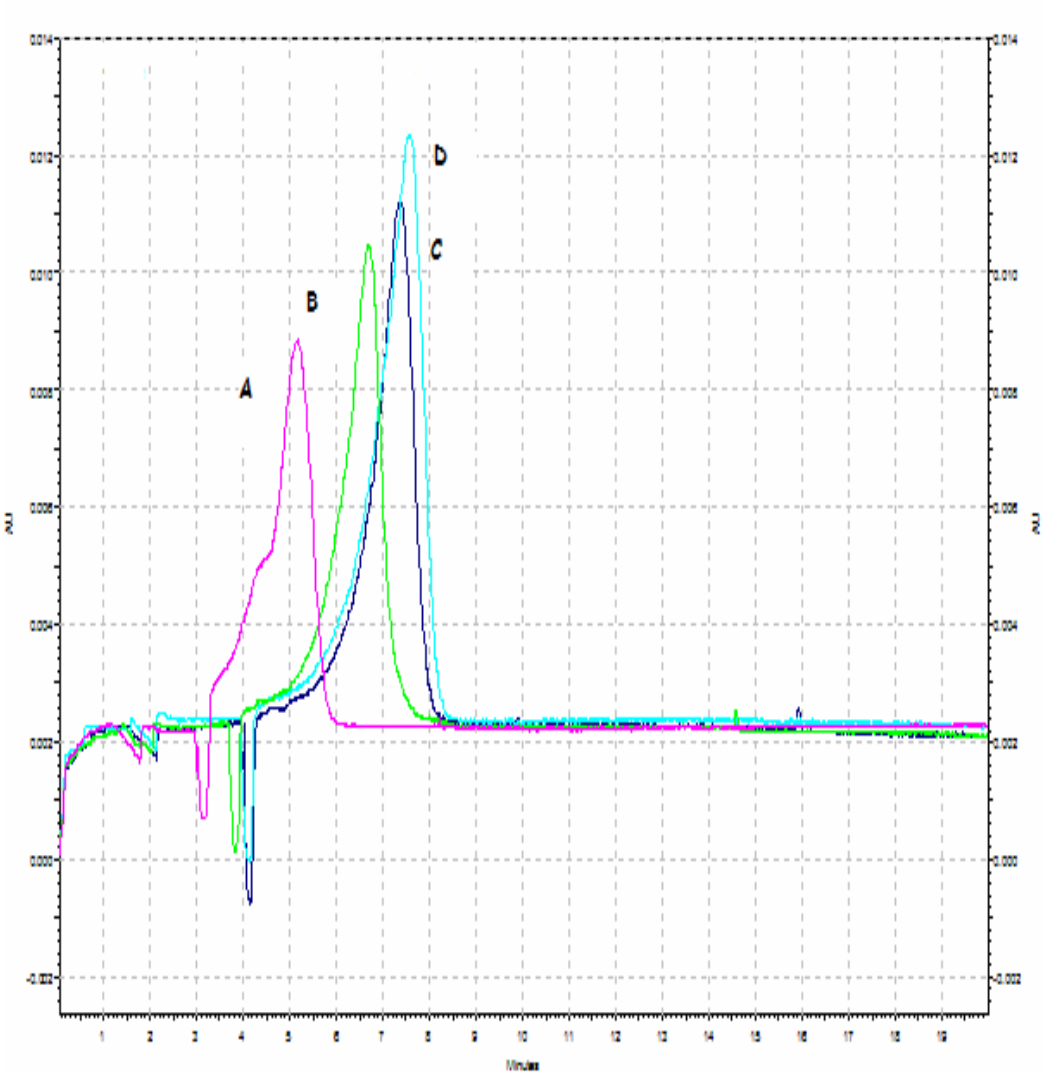


Figure 17 Representative capillary electropherograms: (A) monomethoxypoly (ethylene) glycol activated by succinimidyl succinate (10 mg/ml), (B) PEGylated HD-AdLacZ, (C) unmodified HD-AdLacZ and (D) HD-Ad after the conjugation reaction without PEG. Samples were diluted 1:2 with sample buffer (20 mM sodium phosphate buffer, pH7.0, 5 mM sodium chloride). Capillary length was 27 cm.

2. Toxicity profiles of PEGylated HD-Ad

2.1 Tissue damage

Serum biochemical markers were measured at various times postinjection (0, 1, 3, 6, 24, 48, 72 and 96 hours) to assess tissue and liver damage of four baboons (*Papioi* sp., 6-7 kg) sedated with ketamine and injected in the left cephalic vein with two different doses of HD-Ad-LacZ and PEG HD-Ad-LacZ: $5x10^{11}$ vp/Kg and $3x10^{12}$ vp/Kg. Within the first hours after the administration, baboons treated with low and high doses of no PEGylated vector, underwent an increase in serum levels of serum aspartate aminotransferase (AST) and Lactate dehydrogenase (LDH), with levels that were two to three times higher in the high dose baboon respect to the low dose baboon. The serum levels returned to within normal limits by 24 hr. An increase of serum levels of AST and LDH was also observed within the first hours after the administration of baboons injected with low and high doses of PEG HD-Ad-LacZ but were one to two and three to five times lower compared to the values of animals treated with the respective dose of HD-Ad-LacZ ***(Fig. 18)***.

These results suggest that liver and tissue damage occurred rapidly after vector exposure. This was relatively mild and transient in the baboons treated with PEGylated vectors.

2.2 Coagulopathy

Markers for disseminated intravascular coagulation (DIC) were measured at various times postinjection. Within the first hours after the injection, prothrombin time (PT) of baboons treated with HD-Ad-LacZ increased from a baseline time, with higher levels in the high dose baboon, and back to baseline level by 24 hour. Serum levels of PT in each of baboons injected with modified vector increased not much in the first time to return quickly at normal values. The baboon treated with low dose of no modified vector, showed an increase of values of the activated partial thromboplastin time (aPTT) that decreased to a nearly normal

values in the subsequent hours, while in the high dose baboons the PPT rose, reaching the major peak at 24 hours and then underwent a rapid decrease. Serum levels of PPT in baboons injected with PEGylated vector, increased until to 24 hours, and then decreased but kept values lower respect to the results reaching with baboons treated with unmodified vector ***(Fig. 19)***.

At 3 hours after vector administration, a modest drop in platelet count was detected in the animal treated with low dose of unmodified HD-Ad; this value rose slightly within six hours, and then had a significant drop. Similar result showed the platelet count of the animal administred with low dose of PEG HD-Ad-LacZ, with a major difference in the values in the first six hours. The platelet count in the high dose baboons treated with unmodified vectors was no different than those from animal treated with modified vector. In the first hours both animals showed a drastic drop in platelet count, but the values returned to baseline only for the baboon treated with PEGylated vector by 72 hours postinjection ***(Fig.20)***. These results provide evidence that systemic administration of HDAd resulted in DIC, with a decrease of platelets and increase of PT and PTT, while the PEGylation alleviates vector-induced thrombocytopenia after systemic administration and prevent the prolongation of coagulation time.

2.3 Acute inflammation

Several markers for activation of the acute inflammatory response were measured at various times postinjection. Similar to conditions of acute infection and inflammation, all baboons administred with unmodified vector showed a rapid increase in white blood cell counts (WBC) shortly after vector injection ***(Fig.21)***. In the first 6 hours after injection, both baboons showed an increase of granulocytes, while the lymphocytes, after an initial decrease, increased and rise a major peak 48 hours after vector administration. For the animals treated with PEGylated vector, the WBC initially decreased, but then increased reaching the higher values at 6 hours; the values were lower in the high dose baboons. The granulocytes, for both animals injected with modified vector, increased within 3

hours, while the lymphocytes decreased to increase between 24 and 72 hours ***(Fig.22)***.

Serum samples were collected 6 h after administration of each vector and analyzed for interleukin 6 (IL-6), interleukin 12 (IL-12) and tumor necrosis factor (TNF-α), markers commonly associated with activation of the innate immune response against recombinant adenoviral vectors. A single 6 h time point was selected because, in a mouse model, these cytokines peak approximately 6 h after vector administration and rapidly decline to baseline values by 24 h.

IL-6, IL-12, TNF-α increased rapidly after injection of vector in animals treated with HD-Ad-LacZ and were three, two and seven times higher in the baboons treated with high dose respect to the baboons administred with low dose. In the animal treated with low dose of PEGylated vector IL-6 and TNF-α were absent, while the values of IL-12 were lower compared with serum level of IL-12 in the baboons injected with unmodified vector. The baboons treated with high dose of PEG HD-Ad-LacZ, showed IL-6 and IL-12 secretion with values lower respect to the values of unmodified vectors, while TNF-α was absent ***(Fig.23)***.

Taken together, these results suggest that an acute inflammatory response was provoked as a consequence of systemic unmodified vector administration, the severity of which appeared dose dependent but PEGylation may adequately dampen the innate response in a manner that reduces acute inflammation.

2.4 In vivo performance of PEGylated HD-Ad

X-Gal histochemistry was performed on tissues to determine the degree and distribution of vector transduction. There was a different transgene expression *in vivo,* indeed histochemical liver section of baboons treated with $5x10^{11}$ vp/kg of unmodified revealed that many hepatocytes stained positive for *β*-galactosidase but significantly more transduction was observed in histochemical liver section of baboons treated with low dose of PEGylated vector ***(Fig.24)***.

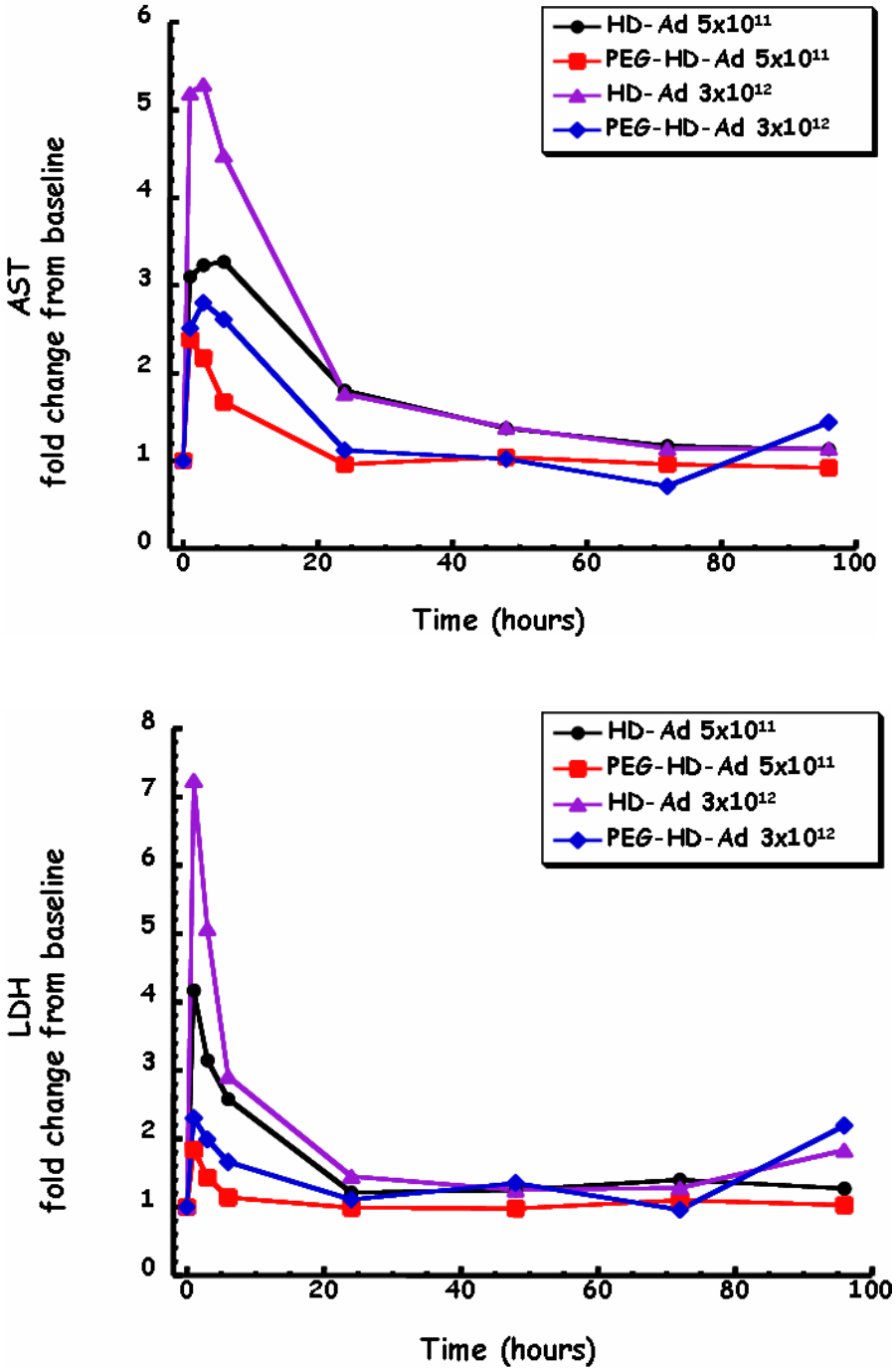


Figure 18 Serum levels of AST and LDH: The graphs shown serum levels of AST and LDH in the baboons treated with low and high dose of unmodified and modified vector. The tissue and liver damage was reduced in the baboons treated with PEGhilated vector.

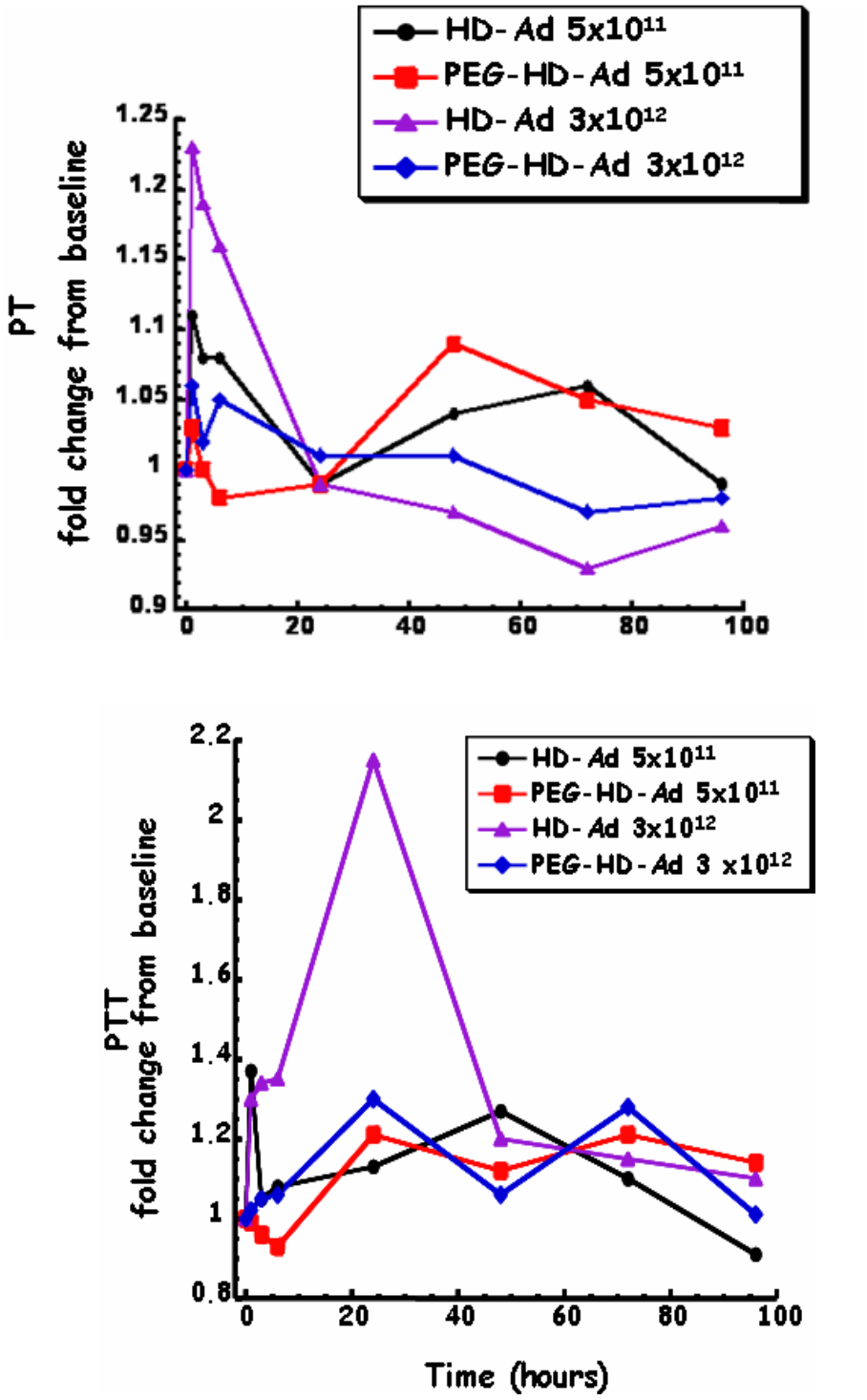


Figure 19 Serum levels of PT and PTT: The graphs shown serum levels of PT and PTT in the baboons treated with low and high dose of unmodified and modified vector. The prolongation of coagulation time was reduced in the baboons treated with PEGhilated vector.

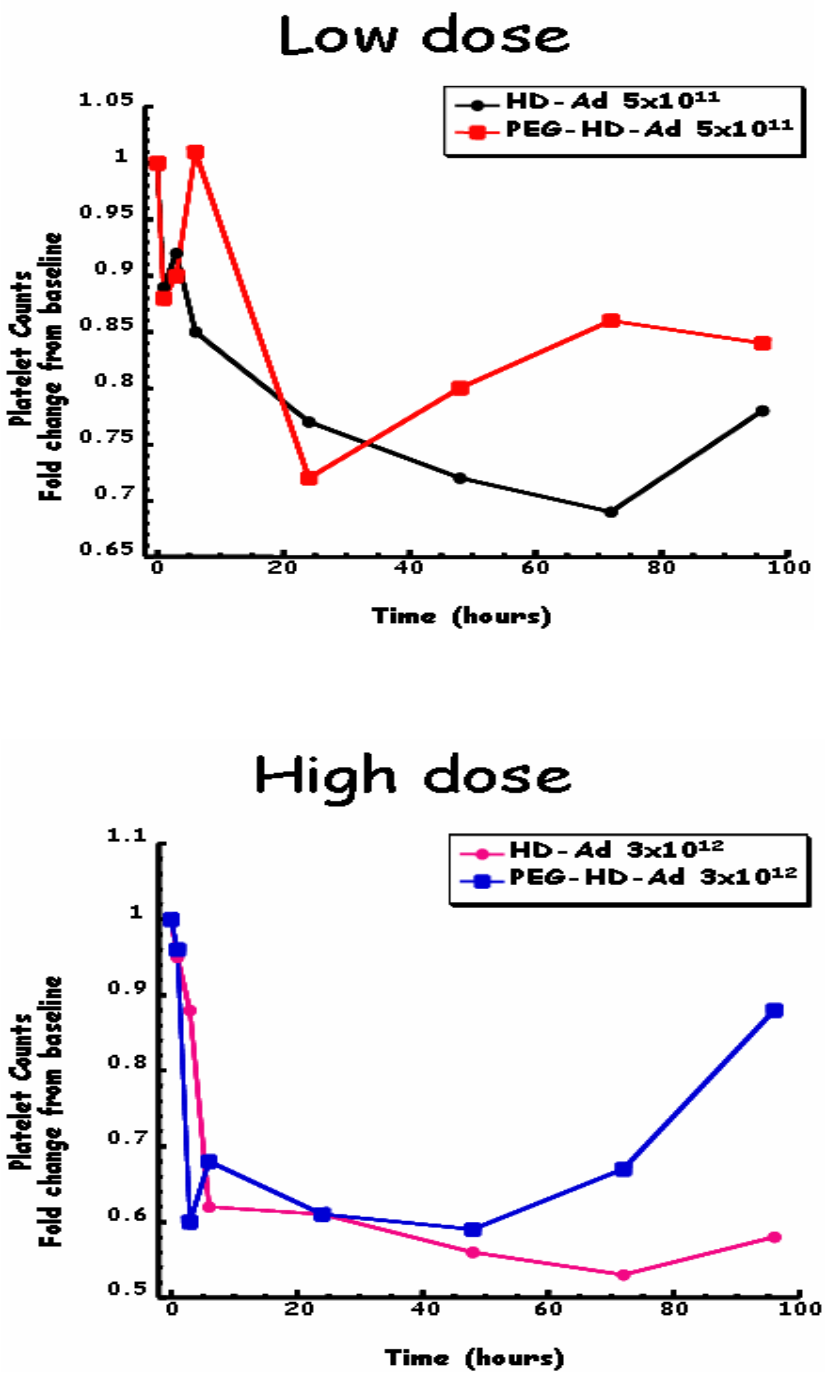


Figure20. Platelet count: The graphs show the platelet count in the baboons treated with low and high dose of unmodified and modified vector. PEGylation of helper-dependent adenovirus prevents vector-induced thrombocytopenia after systemic delivery.

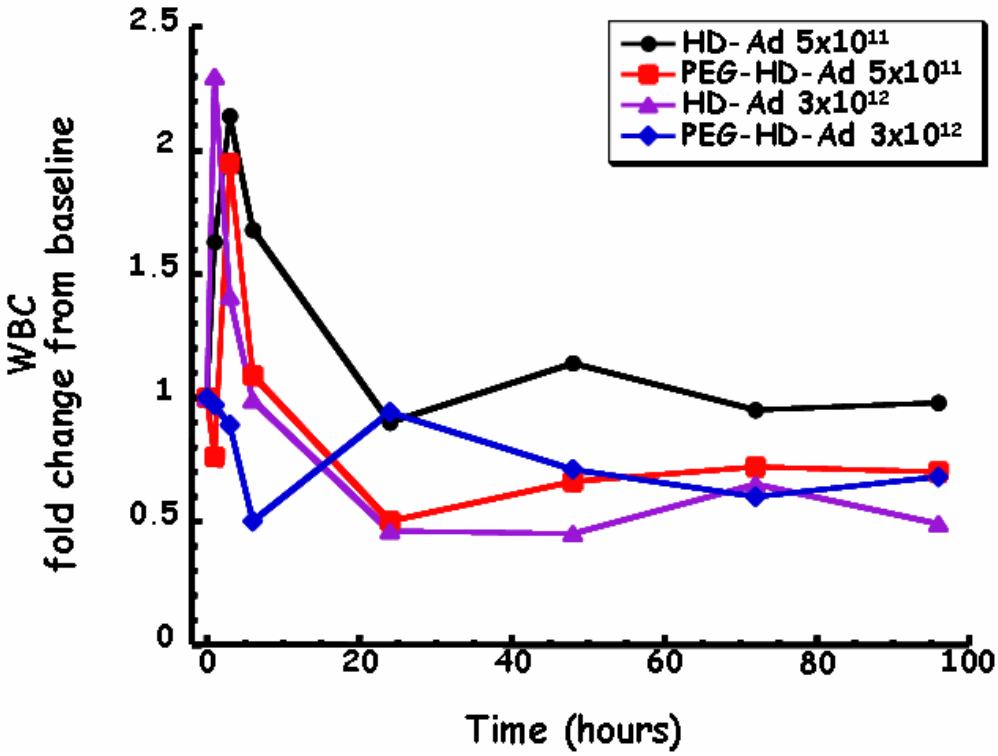


Figure 21 Serum levels of WBC: The graphs shown serum levels of WBC in the baboons treated with low and high dose of unmodified and modified vector. Similar to conditions of acute infection and inflammation, all baboons administred showed a rapid increase in WBC shortly after vector injection

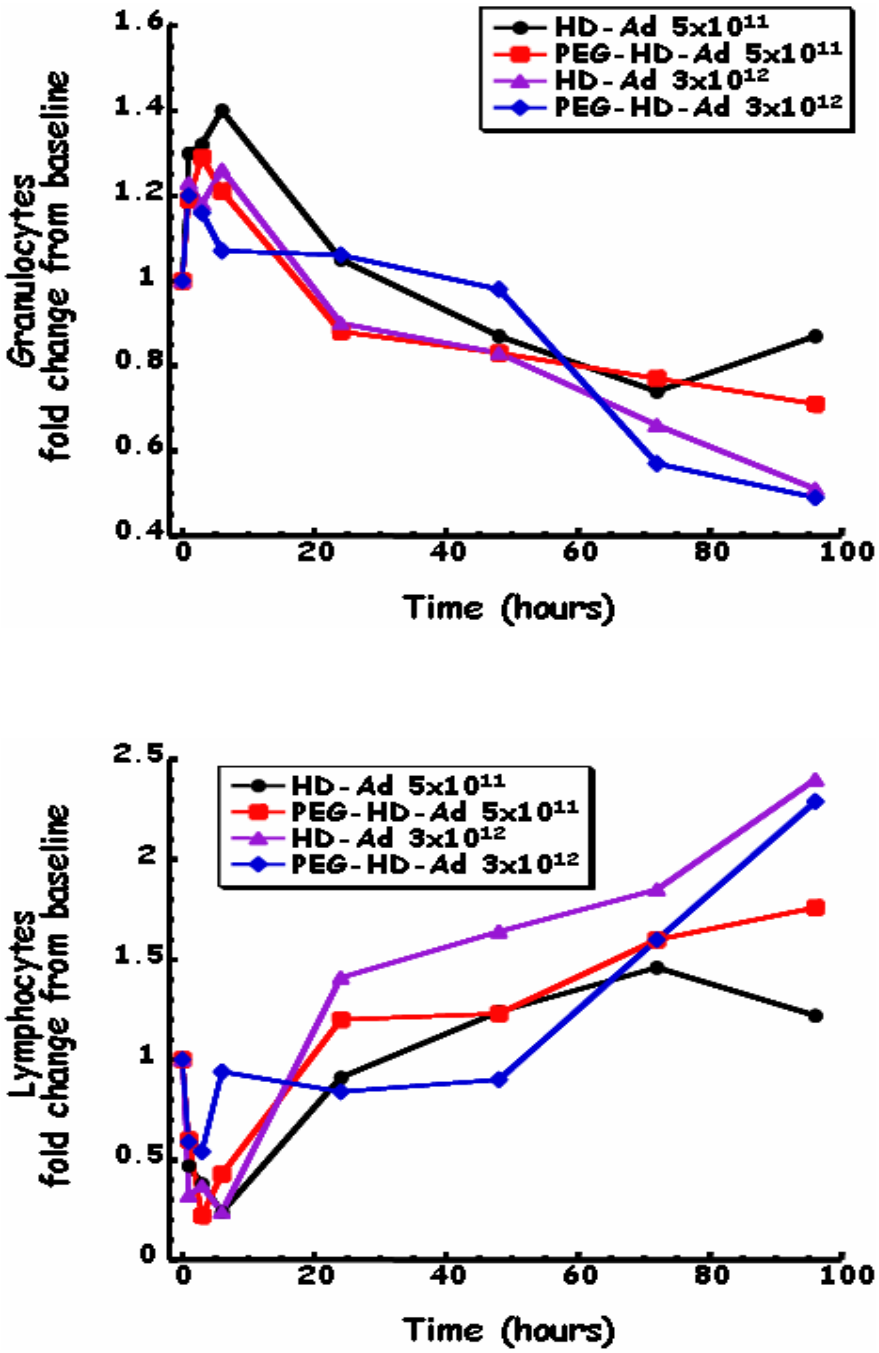
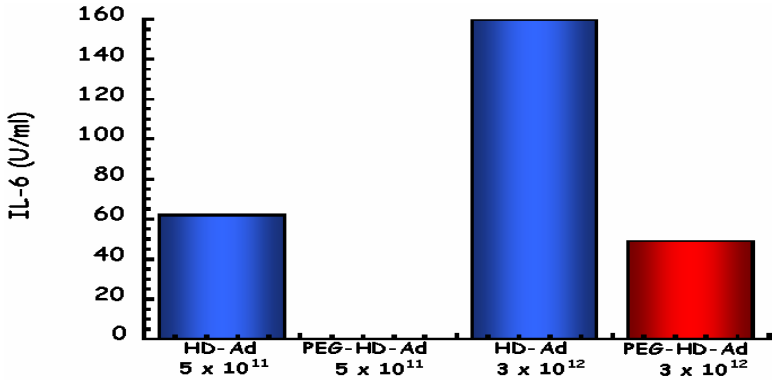
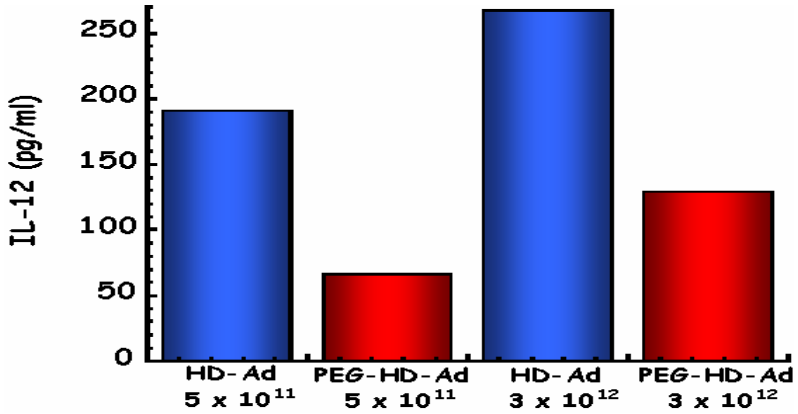


Figure 22 Serum levels of granulocytes and lymphocytes: The graphs shown serum levels of granulocytes and lymphocytes in the baboons treated with low and high dose of unmodified and modified vector. However, the modified vector provoked a low acute toxicity.





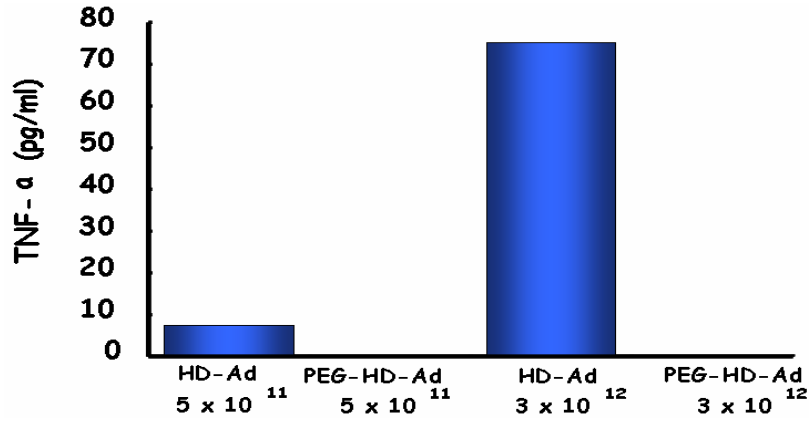
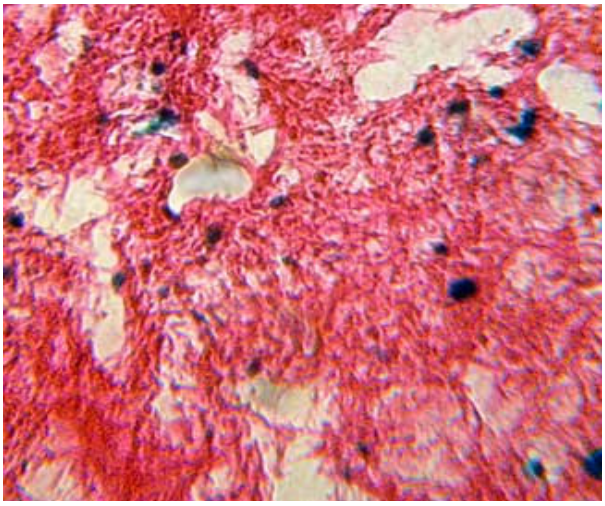


Figure 23 Serum levels of proinflammatory citokynes: The graphs shown serum levels IL-6, IL-12, TNF-α in the baboons treated with low and high dose of unmodified and modified vector. PEGylation of recombinant Ad reduces serum levels of pro- inflammatory cytokines

A



B

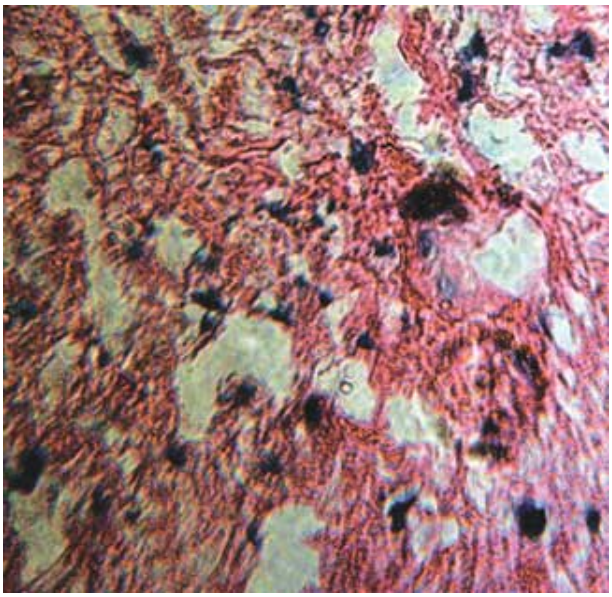


Figure 24 LacZ staining of frozen liver section: Liver section from baboons injected with (A) unmodified or (B) PEGylated HDAdLacZ, at 5 x10 ^{11} vp/ were stained. Surprisingly, the baboon treated with PEGylated vector showed more hepatic transduction respect to the baboon treated with the unmodified vector.

3. Biodistribution of PEGylated vectors in vivo

Four baboons were administered with two different doses of HD-AdLacZ and PEGHD-AdLacZ intravenously through cephalic vein injection. 96 hours later, the animals were sacrificed, various organs were harvested (liver, gonads, intestine, spleen, kidney, stomach, heart, pancreas, brain, lungs and skeletal muscle) and DNA was extracted to determined the levels of vector DNA (vector copy number/ng genomic DNA) by real-time PCR.

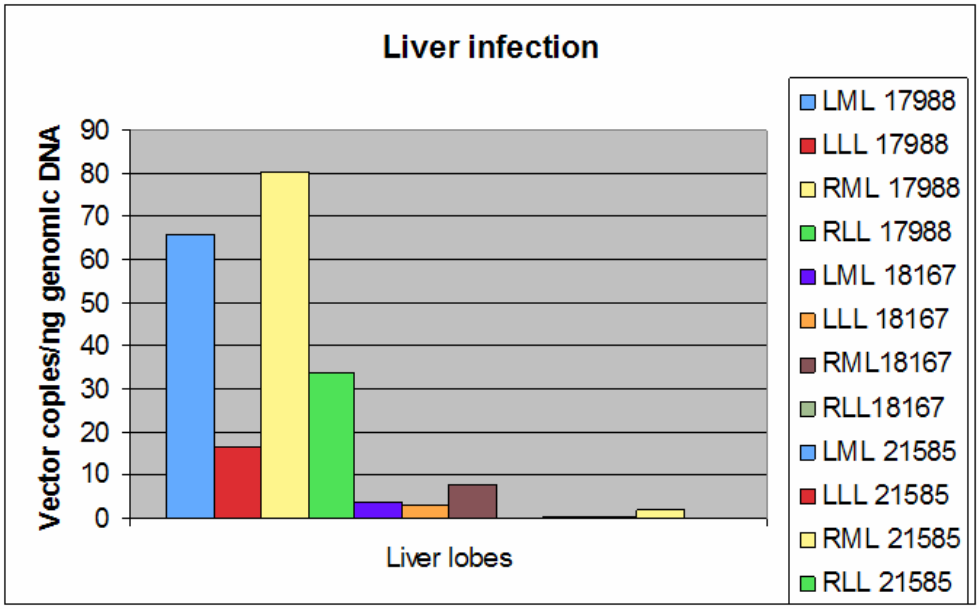
The liver lobes showed different infectivity levels: the left median lobe (LML) and the right median lobe (RML) of baboons treated with low dose of unmodified vector were infected about sixty and seventy times more than the some lobes of baboons treated with PEGylated vector; in the left liver lobe and the right liver lobe of baboons administred with low dose of HD-AdLacZ was observed an infectivity about ten and thirty times higher respective to baboon injected with the some dose of PEG-HD-AdLacZ. The high dose of PEGylated vector didn't present signs of infectivity in the liver lobes.

Surprisingly, the infectivity in the spleen of baboons treated with low dose of modified vector was eight times higher compared to the infectivity in the same organ of all other animals ***(Fig.24)***

In the kidneys, the infectivity of modified and unmodified low dose vectors appeared to be higher than the infectivity of high dose vectors, but it is not very high.

Finally, the lungs of baboons treated with low dose of unmodified vector, were infected two and one times more than those of the baboons injected with low dose of PEGylated vector. The high dose of modified vector didn't infect the lungs ***(Fig.25)***. Like to the kidney, the infectivity levels were very low.

This data suggest that the virus infectivity was very poor in an independent dose manner.



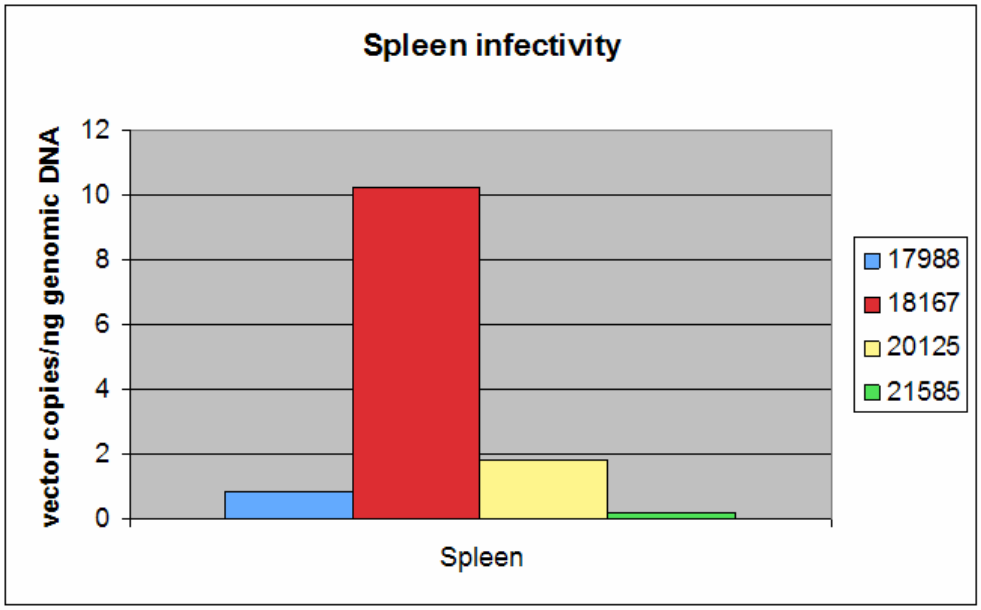
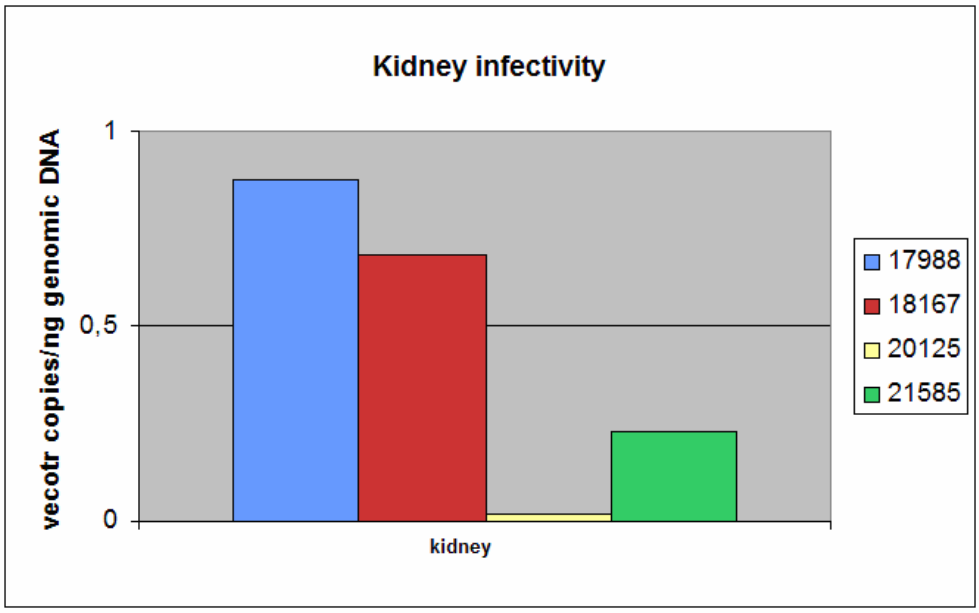


Figure 24 Real-time PCR : The graphs show the vector infectivity in lobe of liver and in the spleen for the baboon treated with unmodified low dose of vector (17988), with modified dose of vector (18167), with unmodified high dose of vector (20125), with modified low dose of vector (21585).



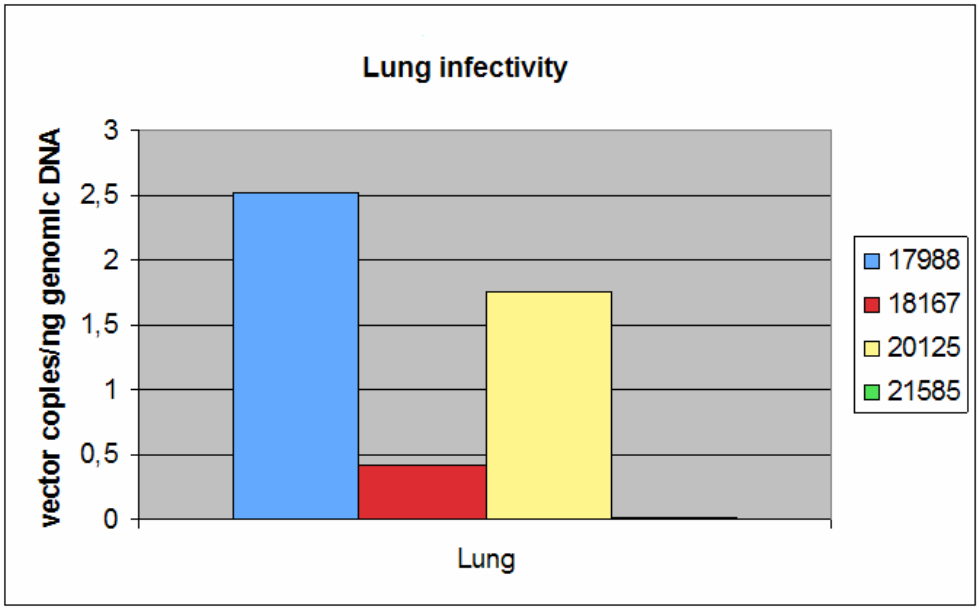


Figure 25 Real-time PCR : The graphs show the vector infectivity in kidney and lung for the baboon treated with unmodified low dose of vector (17988), with modified dose of vector (18167), with unmodified high dose of vector (20125), with modified low dose of vector (21585)

CHAPTER IV

DISCUSSION

Gene therapy is the insertion of genes into an individual's cells and tissues to treat, especially hereditary disease in which a defective mutant allele is replaced with a functional one. In most gene therapy studies, a "normal" gene is inserted into the genome to replace an "abnormal," disease-causing gene, using viral or non viral vector to treat the pathology.

Adenoviruses are robust vectors for *in vivo* gene delivery because of some important reasons : (a) many human and animal adenoviruses are non-pathogenic for their natural hosts, (b) a variety of both proliferating and quiescent cell types, such as epithelial cells, fibroblasts, hepatocytes, endothelial cells and stromal cells, can be infected with Ad vectors, (c) Ad vectors can be grown to very high titers that offer a means to infect a large number of target cells, and (d) replication-competent ((E3) –deleted vectors), conditional replication-competent (vectors in which E1A is under the control of a tissue- or cancer antigen-specific promoter), replication-defective (E1, E1 and E3, E2, E4, E2 and E4, or E1, E2 and E4-deleted vectors) and helper-dependent (vectors in which the majority of Ad genome is deleted) Ad vectors can easily be generated. While potent, Ad is limited by its immunogenicity and liver toxicity when delivered in high doses in rodents, in nonhuman primates and in humans.

Scientists working on Ad vectors for gene therapy learnt a bitter lesson on September 17, 1999, when 18-year-old Jesse Gelsinger died after receiving a very high dose (3.8×10^{13} particles) of Ad vector to treat the ornithine transcarbamylase (OTC) disorder, a rare genetic defect in the liver that renders the body unable to clear ammonia from the bloodstream.. The death of the young patient, gives the biomedical community pause for thought, but it does not change the fact that gene therapy is a relevant and important area of investigation that is

ready for testing in carefully designed human trials. So, this tragedy and a number of subsequent animal inoculation studies demonstrated that higher vector doses invariably lead to hepatotoxicity and acute inflammatory response mainly due to activation of innate immunity.

Significant improvement in the safety and efficacy of Ad-based vectors came with the development of helper-dependent adenoviral vectors, which are deleted of all viral coding sequences. HDAds retain the advantages of FGAds, including high-efficiency *in vivo* transduction and high-level transgene expression. However, owing to the absence of viral gene expression in transduced cells, these HDAds are able to mediate high-level, long-term transgene expression in the absence of chronic toxicity. In addition, because the vector genome exists episomally in transduced cells, the risks of germ line transmission and insertional mutagenesis leading to oncogenic transformation are negligible. Moreover, the deletion of the viral sequences permits a large cloning capacity of ~37 kb, allowing for the delivery of whole genomic loci, multiple transgenes, and large *cis*-acting elements to enhance, prolong, and regulate transgene expression.

The main problem with HD-Ad vectors are the helper virus contamination and the activation of innate immune response trigger by viral capsid proteins, but this is lower the cellular immune response.

Covalent attachment of polymers such as polyethylene glycol (PEG) to Ad capsid has been shown to curtail antibody-mediated virus neutralization. Such modifications are also expected to elude innate immunity since they will potentially mask the molecular patterns on the viral capsid with little or no effect on virus infectivity. Consistent with this, monomethoxypolyethylene glycol conjugation of Ad vector lead to reduced innate immunity and improved therapeutic index in mice when compared to unmodified Ad vectors.

Based on these considerations, we decided to conduct a study to evaluate the toxicity and biodistribution of HD-Ad and PEG-HD-Ad in nonhuman primates.

For the purpose we produced, a helper dependent adenoviral vector contains an MCMV-LacZ expression cassette (HD-AdΔ28E4LacZ). The preps of viruses were produced in 116 cell line that can also grow in suspension, rendering large-scale HDAd production significantly more efficient and simpler, as well as less

costly and labor intensive. We amplified the vector by serial coinfections of 60-mm dishes of 116 cells with 10% of the crude lysate from the previous passage and virus helper AdNG163R-2 for serial passages (P0 to P3). For P4, we coinfected 116 cells with 10% of the crude serial passage 3 lysate in two 150-mm dishes and performed large-scale HDAd production in 3 liters of 116 cells coinfected with 100% of the crude lysate from the 150-mm dish of serial passage 4 and AdNG163R-2.

Our system for producing HDAds represents an improvement over other methods in terms of simplicity and efficiency as well as vector yield and purity. However, it should be stressed that direct comparisons between different systems are difficult due to both fundamental differences in the component reagents (producer cell line, HV, and HDAd backbones) and especially the procedural variations used for HDAd production. So, we produced a virus with a safety and efficacy that can be used meaningful preclinical studies in large animal models and perhaps ultimately for clinical applications.

After virus production we PEGylated a part of the vector using poly (ethylene glycol) and then we administred it at four baboons in two different doses of unmodified and PEGylated vector a low dose (5 $x10^{11}$ vp/kg) and high dose (3x 10^{12} vp/kg) determined the acute clinical, pathological, and biochemical consequences.

Biochemical evidence of liver and tissue damage in the form of elevated levels of AST and LDH we observed in all animals treated, with higher levels in the animal administred with the unmodified vector, at the same time points postinjection.

Indeed, although evidence of DIC we noted in all baboons, with prolongation of PT and aPTT, and decreases in platelets, these abnormalities were generally relatively mild and transient in the animals treated with PEGylated vector and more pronounced in the baboons' administred with unmodified vector.

All animals exhibited evidence of innate inflammatory immune response activation. We observed an elevation of IL-6, IL-12 and TNF-α, and we note that PEGylation significantly reduced cytokine production but did not ablate it. This may be due to the fact that the polymer did not completely cover the virus capsid.

Our results show that the severity of HDAd-mediated acute toxicity is dose dependent but it can be reduced by PEGylated vectors. The Real -Time PCR data showed different organs infectivity with modified and unmodified vector: it is dose independent.

So, we have found that combining PEGylation and HD-Ad technologies significantly reduces cytokine secretion, serum transaminases and alleviates vector-induced thrombocytopenia after systemic administration, leading to a significantly improved therapeutic index of adenoviral gene transfer. This suggests that PEGylation of HD-Ad vectors may be appropriate for their safe and efficient use in the clinic.

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