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Current Strategies and Future Directions for Eluding Adenoviral Vector Immunity

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Abstract

Adenoviral (Ad) vectors can efficiently transduce a broad range of cell types and have been used extensively in preclinical and clinical studies for gene delivery applications. The presence of preexisting Ad immunity in the majority of human population and a rapid development of immune response against the Ad vector backbone following the first inoculation with the vector have impeded clinical use of these vectors. In addition, a number of animal inoculation studies have demonstrated that high systemic doses of Ad vectors invariably lead to initiation of acute inflammatory responses. This is mainly due to activation of innate immunity by vector particles. In general, vector and innate immune responses drastically limit the vector transduction efficiency and the duration of transgene expression. In order to have a predictable response with Ad vectors for gene therapy applications, the above limitations must be overcome. Strategies that are being examined to circumvent these drawbacks of Ad vectors include immunosuppression, immunomodulation, serotype switching, use of targeted Ad vectors, microencapsulation of Ad vectors, use of helper-dependent (HD) Ad vectors, and development of nonhuman Ad vectors. Here we review the current understanding of immune responses to Ad vectors, and recent advances in the strategies for immune evasion to improve the vector transduction efficiency and the duration of transgene expression. Development of novel strategies for targeting specific cell types would further boost the utility of Ad vectors by enhancing the safety, efficacy and duration of transgene expression.

1. ADVANTAGES OF ADENOVIRAL VECTORS FOR GENE THERAPY

Adenoviral (Ad) vectors have been the focus of considerable interest in the last few years for their potential applications as delivery vehicles for human gene therapy [Alemany *et al.* 2000; Bramson *et al.* 1995; Chuah *et al.* 2003; Curiel 2000; Hitt *et al.* 2000; Liu *et al.* 2002; Sadeghi *et al.* 2005; St George 2003]. Results of animal studies and clinical trials in humans for cancer therapy and other metabolic disorders using human Ad (HAd) vectors are encouraging [Akbulut *et al.* 2003; Ambar *et al.* 1999; Emtage *et al.* 1998; Parks *et al.* 1999; Trudel *et al.* 2001; Wen *et al.* 2001]. Some of the important reasons for the choice of Ad vectors for gene therapy are: (i) many human and animal adenoviruses are non-pathogenic for their natural hosts, (ii) 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, (iii) Ad vectors can be grown to very high titers that offer a means to infect a large number of target cells, and (iv) replication-competent (e.g., early region (E) 3 (E3) -deleted vectors), conditional replication-competent (e.g., vectors in which E1A is under the control of a tissue- or cancer antigen-specific promoter), replication-defective (e.g., E1, E1 & E3, E2, E4, E2 & E4, or E1, E2 & E4-deleted vectors) and helper-dependent (e.g., vectors in which the majority of Ad genome is deleted) Ad vectors can easily be generated. Moreover, the

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absence of germ-line transmission of Ad vectors in mice highlights one of the safety aspects of HAd vectors [Paielli *et al.* 2000]. The E1- or E1 and E3-deleted vectors are routinely known as the first generation Ad vectors, and vectors having the deletion of E2 and/or E4, in addition to E1 or E1 and E3 deletion are called the second generation Ad vectors. Helper-dependent (HD)-Ad (HD-Ad) vectors are also referred as gutless, gutted, or high-capacity vectors and can be classified as the third generation Ad vectors.

2. SIGNIFICANCE OF VECTOR IMMUNITY IN GENE THERAPY

More than 50 different serotypes of HAd are known to infect humans. Due to ubiquitous nature of HAd, a majority of the human population is exposed to adenoviruses leading to the development of an HAd-specific immune response [Harvey *et al.* 1999]. The preexisting vector immunity is serotype-dependent and some HAd serotypes are less prevalence than others. Preexisting immunity against a particular serotype will significantly reduce the uptake of the homologous HAd vector. Currently, most HAd vectors are based on HAd serotype 5 (HAd5). The E1-deleted replication-defective HAd vectors are capable of expressing viral early and late genes at a magnitude sufficient to stimulate cellular and humoral immune responses [Dai *et al.* 1995; Elkon *et al.* 1997; Kafri *et al.* 1998; Yang *et al.* 1994; Yang *et al.* 1995]. HAd-specific neutralizing antibodies are directed against the viral capsid components [Toogood *et al.* 1992], and significantly inhibit the virus uptake following readministration of the same vector [Dong *et al.* 1996; Moffatt *et al.* 2000; Sailaja *et al.* 2002; Walter *et al.* 1996]. The cellular immune response, mediated through HAd5-specific CD8+ T cells, eliminates the target cells expressing viral and transgene products. This causes rapid loss of transgene expression in Ad vector-inoculated experimental animals [Crystal 1995].

It has been shown that in addition to the E1 deletion, E2A, E2B and E4 deletions resulted in minimal Ad late gene expression but without a drastic improvement in the duration of transgene expression [Engelhardt *et al.* 1994; Kafri *et al.* 1998; Yang *et al.* 1995]. Subsequently, HD-Ad vectors that have a large portion of the genome deleted were developed [Fisher *et al.* 1996; Kochanek *et al.* 1996; Morsy *et al.* 1998; Parks *et al.* 1996]. Inoculation of animals with an HAd vector having all viral genes deleted resulted in improved safety and prolonged expression of the transgene [Kochanek *et al.* 1996; Morsy *et al.* 1998; Parks *et al.* 1999]. However, significant levels of humoral and cellular immunity were elicited in HD-Ad inoculated animals indicating that expression of viral proteins was not essential for the induction of immune responses [Kafri *et al.* 1998]. Therefore, the first inoculation with any type of Ad vector would result in varying degrees of vector-specific immune response [Hackett *et al.* 2000; Kass-Eisler *et al.* 1996]. Since a number of inoculations with the vector containing the transgene may be needed for most gene therapy applications (Fig. 1), it is important to develop strategies that could effectively elude immunity to the vector.

3. INDUCTION OF INNATE IMMUNE RESPONSE AND TOXICITY BY AD VECTORS

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 HAd vector containing the ornithine transcarbamylase (OTC) gene. 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. Innate immune response is activated following recognition of molecular patterns on Ad capsid by pattern recognition receptors on macrophages (mφ) and dendritic cells (DC), resulting in activation of multiple signaling pathways such as mitogen-activated protein kinase (MAPK) and nuclear factor (NF)-κB pathways that augment expression of several proinflammatory cytokines and chemokines [Bruder *et al.* 1997; Lieber *et al.* 1997;

Muruve *et al.* 1999; Shifrin *et al.* 2005] (Fig. 2). Multiple inflammatory cytokines and chemokines including interleukin (IL)-6, IL-8, IL-12, tumor necrosis factor (TNF)-α, interferon-&;ambda;, RANTES (Regulated upon Activation, Normal T-cell Expressed and Secreted), interferon-inducible protein 10 (IP-10), macrophage inflammatory protein (MIP)-1β, and MIP-2 are expressed following Ad vector administration in a dose-dependent manner [Elkon *et al.* 1997; Zaiss *et al.* 2002].

3.1. Implication of Route of Inoculation on Innate Immunity

Following intravenous (i.v.) inoculation, HAd vectors are in general taken up by the reticuloendothelial cells in the liver, leading to a rapid induction of an innate immune response. Intraportal infusion of an E1-deleted HAd5 vector (7.5×10^{12} particles/kg) in nonhuman primates resulted in acute activation of mφ and DC followed by considerable apoptosis of splenocytes and hepatocytes due to activation of innate immunity by viral capsid proteins [Schnell *et al.* 2001]. However, vector doses up to 5×10^{12} particles/kg usually lead to only limited hepatitis suggesting that vector toxicity could be diminished by lowering the vector dose per inoculation. In another study, i.v. inoculation of mice with a HAd5 vector (2×10^{11} genomes/mouse) led to acute inflammatory response characterized by high levels of IL-6 and IL-12 expression due to preferential activation of mφ and DC [Zhang *et al.* 2001].

The i.v. delivery of Ad vectors also lead to activation of endothelial cells as detected by expression of phosphorylated Akt/PKB kinase, activated endothelial nitric oxide synthase (eNOS), and nitrotyrosine due to interaction of viral particles with Kupffer cells [Schiedner *et al.* 2003a]. Conserved arg-gly-asp (RGD) motifs of the adenovirus capsid appeared to be important for efficient vector transduction and endothelial cell activation [Liu *et al.* 2003]. In rhesus monkeys, i.v. inoculation of HAd vector induced thrombocytopenia by enhancing *in vivo* platelet clearance [Wolins *et al.* 2003]. Similarly, a baboon inoculated with an E1-deleted HAd vector developed acute symptoms, decreased platelet counts, increased liver enzymes, injury to the vascular endothelium, and became moribund at 48 h post-inoculation [Morral *et al.* 2002]. These studies underscore the importance of induction of a strong innate immune response following Ad vector administration in mediating an acute inflammatory reaction.

In a subcutaneous mouse mammary tumor model, pre-immunization with an HAd5 vector resulted in significantly reduced transgene expression in the tumor and normal tissues, however, the inhibition was more in the liver than in the mammary tumor [Bramson *et al.* 1997; Vlachaki *et al.* 2002]. Increasing the vector dose by 10- to 100-fold restored the level of transgene expression in preimmunized mice, but higher vector doses (2×10^{11} virus particles or more per inoculation) also led to significantly higher hepatotoxicity compared to naïve animals. Readministration of a second vector dose was associated with the same degree of toxicity as the first vector, but prompted a much more vigorous neutralizing antibody response [Nagao *et al.* 2001; Nunes *et al.* 1999]. Increased mortality was observed when pre-immunized mice were inoculated systemically with a high dose of Ad vector [Varanvski *et al.* 2005]. Pre-exposure failed to inhibit induction of pro-inflammatory cytokines but tissue toxicity was reduced. In cirrhotic rats, the biodistribution of HAd vectors shifted from the liver to the lungs due to the presence of pulmonary intravascular mφ [Smith *et al.* 2004b]. High doses of HAd vectors in cirrhotic rats not only upregulated TNF-α and IL-6 expression, but also led to markedly prolonged coagulation times, and resulted in fatal pulmonary hemorrhagic edema [Smith *et al.* 2004a]. Cellular gene expression in response to wild type Ad, Ad vectors, or empty Ad particles was similar [Stilwell *et al.* 2003], suggesting the importance of the viral capsid proteins in mediating vector toxicity without viral gene expression. Additionally, it has been shown that intramuscular (i.m.) inoculation but not i.v. inoculation resulted in prolonged and sustained transgene expression and effective evasion of preexisting Ad immunity [Maione *et al.* 2001].

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 mφ play an important role in the induction of innate immune response following vector inoculation, depletion of mφ and DC 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 mφ 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 mφ prior to intratracheal (i.t.) 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 mφ 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. Covalent Modification of Ad Capsid

Alteration of the immunodominant epitopes of the Ad capsid was also helpful in evading Ad immunity [Roy *et al.* 1998]. Covalent attachment of polymers such as polyethylene glycol (PEG) [Croyle *et al.* 2000; Croyle *et al.* 2002; Lanciotti *et al.* 2003; O'Riordan *et al.* 1999] or N-(2-hydroxypropyl) methacrylamide (HPMA) [Fisher *et al.* 2001] 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, mono-methoxypolyethylene glycol conjugation of Ad vector lead to reduced innate immunity and improved therapeutic index in mice when compared to unmodified Ad vectors [Geest *et al.* 2005].

PEGylation of vectors substantially lowered innate Il-6 responses by HD-Ad as well as first-generation Ad vectors without significantly affecting transduction efficiency [Mok *et al.* 2005]. These reduced innate responses paralleled reductions in vector uptake by mφ *in vitro* and Kupffer cells *in vivo*. In addition to demonstrating the possibility of evading vector immunity by covalent modification of HD-Ad vectors, these studies also highlighted the role of nonspecific vector uptake by mφ in inducing innate immunity against Ad [Mok *et al.* 2005]. PEGylation of HD-Ad vectors did not adversely affect *in vitro* and *in vivo* transduction efficiencies but lowered peak serum IL-6, IL-12 and TNF-α levels compared to normal HD-Ad vectors [Croyle *et al.* 2005] suggesting that innate immune response elicited by Ad capsid components is critical in mediating vector toxicity.

4.3. Altering Native Ad Vector and Cell Surface Receptor Interactions

HAd5 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 HAd in families A, C, D, E, and F but not B [Bergelson *et al.* 1997; Roelvink *et al.* 1998; Tomko *et al.* 1997]. In addition, major histocompatibility (MHC) class I α2 domain [Hong *et al.* 1997], heparin sulfate glycosaminoglycan [Smith *et al.* 2003] or sialic acid saccharide [Arnberg *et al.* 2000] may also serve as the primary receptor for HAd.

In addition to these primary receptors, host cell integrins serve as co-receptors for Ad entry [Wickham *et al.* 1993]. The HAd penton base protein interacts with vitronectin-binding integrins, specifically $\alpha_v\beta_3$ and $\alpha_v\beta_5$, 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 HAd penton and $\alpha_v\beta_1$ integrins promotes actin cytoskeletal reorganization via activation of several signaling molecules [Li *et al.* 2001]. Binding of the HAd5 fiber knob to CAR receptor could be effectively prevented with a knob-specific antibody. For targeting HAd5 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. 3). 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; Curiel 1999; Douglas *et al.* 1996; Gu *et al.* 1999; Krasnykh *et al.* 1998]. These modified vectors should be preferentially taken up by the specific cells.

HAd5 fiber knob has been shown to induce DC activation and maturation [Molinier-Frenkel *et al.* 2003]. Virus-induced maturation of DC was significantly reduced when knobless Ad particles were incubated with immature DC. 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 HAd35 capsid and HAd5 fiber knob was enhanced indicating a potential role of the fiber knob in the immunogenicity of HAd5 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; Sumida *et al.* 2005], 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 HAd fiber [Aoki *et al.* 2001; Belousova *et al.* 2002; Biermann *et al.* 2001; Douglas *et al.* 1999; Mizuguchi *et al.* 2001; Nicklin *et al.* 2001], protein IX [Dmitriev *et al.* 2002; 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].

4.4. Vector Microencapsulation

The use of polyethylene glycol-cationic lipid to coat HAd 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 [Aggarwal *et al.* 1999; Bowerstock T.L. *et al.* 1999; Hilbert *et al.* 1999; Mittal *et al.* 2000; Periwal *et al.* 1997]. 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 a HAd5 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 mφ and DC [Lomotan *et al.* 1997]. It appears that alginate microspheres may be an attractive delivery system to target mφ and DC, but there is a need to study the role of these microparticles in modulating the immune response through mφ and DC. Use of bilamellar cationic liposomes to encapsulate HAd vectors also provided protection from preexisting humoral immune responses [Yotnda *et al.* 2002]. Similarly, microsphere-liposome complexes guard HAd 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 vectors 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.

4.5. Use of Alternate HAd Serotypes (Serotype Switching)

Since more than 50 HAd 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 [Kass-Eisler *et al.* 1996; Mack *et al.* 1997; Mastrangeli *et al.* 1996; Parks *et al.* 1999]. Subgroup B Ad, such as HAd3, HAd11, and HAd35, 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 HAd11 and HAd35 makes them promising vectors for *in vivo* applications. Replication-defective HAd35 vectors efficiently transduced human cells and eluded preexisting HAd immunity [Gao *et al.* 2003; Reddy *et al.* 2003; Sakurai *et al.* 2003; Vogels *et al.* 2003]. Similarly, HAd11 based replication-defective vectors have shown expanded tropism [Holterman *et al.* 2004; Stone *et al.* 2005]. HAd35 based replication-defective vector vaccines evaded preexisting HAd5 immunity in mice [Barouch *et al.* 2004] as well as in rhesus monkeys [Shiver *et al.* 2004].

4.6. 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.* 1998; Morral *et al.* 1999; Morsy *et al.* 1998] 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; Kim *et al.* 2001; Morral *et al.* 1999].

HD-Ad vectors also induce vector-specific immune response similar to that generated by E1-deleted HAd [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 [Brunetti-Pierri *et al.* 2004; Stilwell *et al.* 2003], implying the importance of the viral capsid components in vector toxicity. The generation and potential applications of HD-Ad vectors have been reviewed [Ng *et al.* 2002].

It has been demonstrated that HD-Ad vectors could be used for *in utero* gene delivery for long-term transgene expression for genetic disorders such as Duchenne muscular dystrophy (DMD) [Bilbao *et al.* 2005]. Like first-generation HAd vectors, HD-Ad vectors do not integrate into the host cell genome; therefore, vector DNA will be gradually diluted out in dividing cells. In situations where long-term gene expression is desired, such as DMD, vector integration into the host genome will further improve longevity of transgene expression. The hybrid HAd-adeno-associated virus (AAV) vectors could provide nonrandom integration of double-stranded DNA by *ex vivo* or *in vivo* gene delivery [Recchia *et al.* 2004], thereby serving as a continuous source of trans-gene expression without potential systemic toxicity. Alternatively, long-term gene expression can be achieved by using a novel binary HD-Ad-Epstein-Barr virus (HDAd-EBV) hybrid system for stable transfection of mammalian cells [Dorigo *et al.* 2004]. This system consists of a cre-recombinase expressing HD-Ad, and a HD-Ad carrying EBV episome and a transgene flanked by *loxP* sites.

HD-Ad vectors have also been investigated for their application in long-term neurological gene therapy. While, transgene expression from a first-generation Ad vector was completely eliminated following peripheral immune priming, HD-Ad vectors produced sustained transgene expression in the rat brain [Thomas *et al.* 2000]. Even in the presence of anti-HAd immunity, an HD-Ad system resulted in sustained and regulatable transgene expression in the brain [Xiong *et al.* 2006]. It may obviate the need to screen patients for pre-existing vector immunity especially for gene delivery to the brain.

Following i.v. inoculation of HD-Ad vectors, an early expression of inflammatory cytokine and chemokine genes, including IP-10, MIP-2, and TNFα, was induced in the liver in a pattern similar to that induced by first generation HAd vectors [Muruve *et al.* 2004]. HD-Ad vectors also induced the recruitment of CD11b-positive leukocytes to the transduced liver cells within hours of administration [Muruve *et al.* 2004]. While first-generation HAd vectors induced a second phase of liver inflammation, consisting of inflammatory gene expression and CD3-positive lymphocytic infiltrate at 7 days post-transduction, these changes were not detected in the livers of mice receiving HD-Ad beyond 24 h post-transduction [Muruve *et al.* 2004]. In addition, adaptive immune responses generated by HD-Ad vectors was also attenuated in comparison to that of first-generation HAd vectors [Muruve *et al.* 2004].

4.7. Use of Nonhuman Ad Vectors

Since Ad viruses are species-specific, nonhuman Ad are expected to be nonprevalent in humans, and therefore, they evade preexisting HAd immunity. In order to extend the range of Ad vectors that could be used to evade HAd neutralizing immune response, a number of

nonhuman Ad such as bovine Ad type 3 (BAd3) [Mittal *et al.* 1995; Reddy *et al.* 1999b; van Olphen *et al.* 2002], canine Ad type 2 [Hemminki *et al.* 2003; Klonjkowski *et al.* 1997], ovine Ad [Hofmann *et al.* 1999], chimpanzee Ad [Farina *et al.* 2001; Xiang *et al.* 2002], and porcine Ad type 3 [Bangari *et al.* 2004; Reddy *et al.* 1999a] 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; Farina *et al.* 2001; Klonjkowski *et al.* 1997; Mittal *et al.* 1995; Rasmussen *et al.* 1999]. Since HAd5-, BAd3- and PAd3-specific neutralizing antibodies do not cross-neutralize [Moffatt *et al.* 2000], it is expected that sequential administration of HAd5, BAd3 and PAd3 would effectively evade the vector-specific neutralizing immune response. The sera of mice immunized with HAd serotypes 2, 4, 5, 7, and 12 did not neutralize chimpanzee Ad [Farina *et al.* 2001] indicating the utility of such vectors in evading HAd 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. The progress in design and construction of various nonhuman Ad vectors has been reviewed recently [Bangari *et al.* 2006].

5. CONCLUDING REMARKS

Various Ad vectors seem to have considerable potential for preventive or therapeutic applications where transgene expression for a short duration may be enough for the desired effects, e.g., for developing recombinant vaccines for human and veterinary use and for cancer gene therapy. The use of Ad vectors for gene therapy of genetic disorders will be more challenging since therapeutic gene expression will be required for extended period of time. It should be noted that induction of proinflammatory cytokines and chemokines by Ad vectors might not be a limitation in every situation. On the contrary, it may be advantageous in some situations such as cancer immunotherapy and preventive vaccination.

In addition to vector immunity, the immune response could also be induced against the transgene product in situations where it is recognized as a foreign antigen by the host. This would also adversely affect the persistence of transgene expression. It is known that the E3 gene products are involved in modulating host immune responses to the virus; therefore, inclusion or deletion of one or more E3 genes will have implications in vector immunity. All novel modifications in vector design are required to be tested extensively in experimental animal models to evaluate their usefulness in evading vector immunity and toxicity. The use of transgenic mice will be useful in further evaluating the strategies for evading vector-induced innate and adaptive immune responses and toxicity. For the purpose of expanding the tropism and modifying vector immunity and toxicity, development of nonhuman adenoviral vectors and human-nonhuman chimeric vectors hold considerable potential. Further investigations on various human and nonhuman Ad surface proteins involved in receptor-mediated internalization will also help to develop better vectors with the ability to target specific receptors. The cross-reactivity of cellular immune responses among different Ad needs to be evaluated to develop strategies for eluding vector cellular immunity by sequential administration of human and/or nonhuman Ad vectors. Adaptation of the information gleaned from other viral vector systems, micro- or nano-particle technology, and mechanism/s of induction of innate and adaptive immune responses will certainly facilitate further improvement in Ad vector design and delivery.

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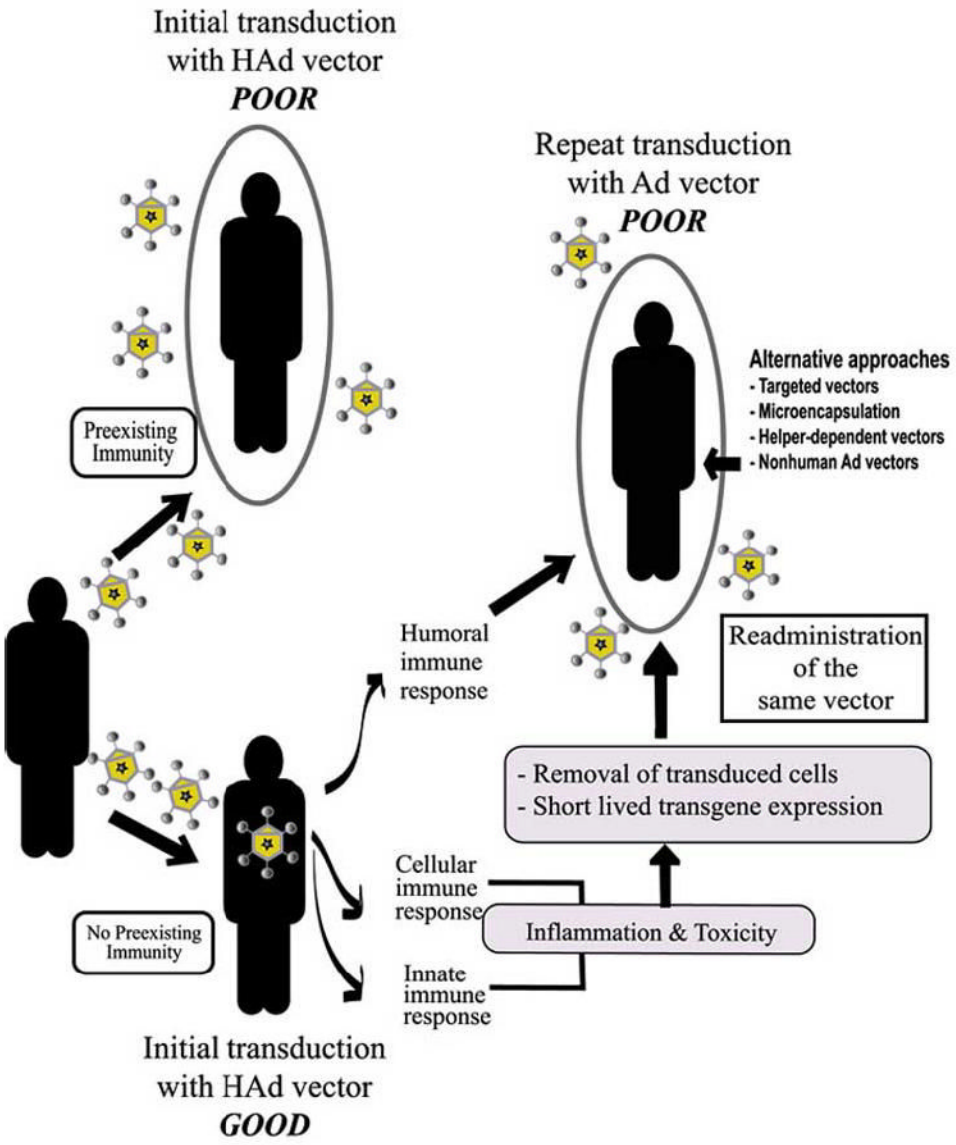

Fig. (1).

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

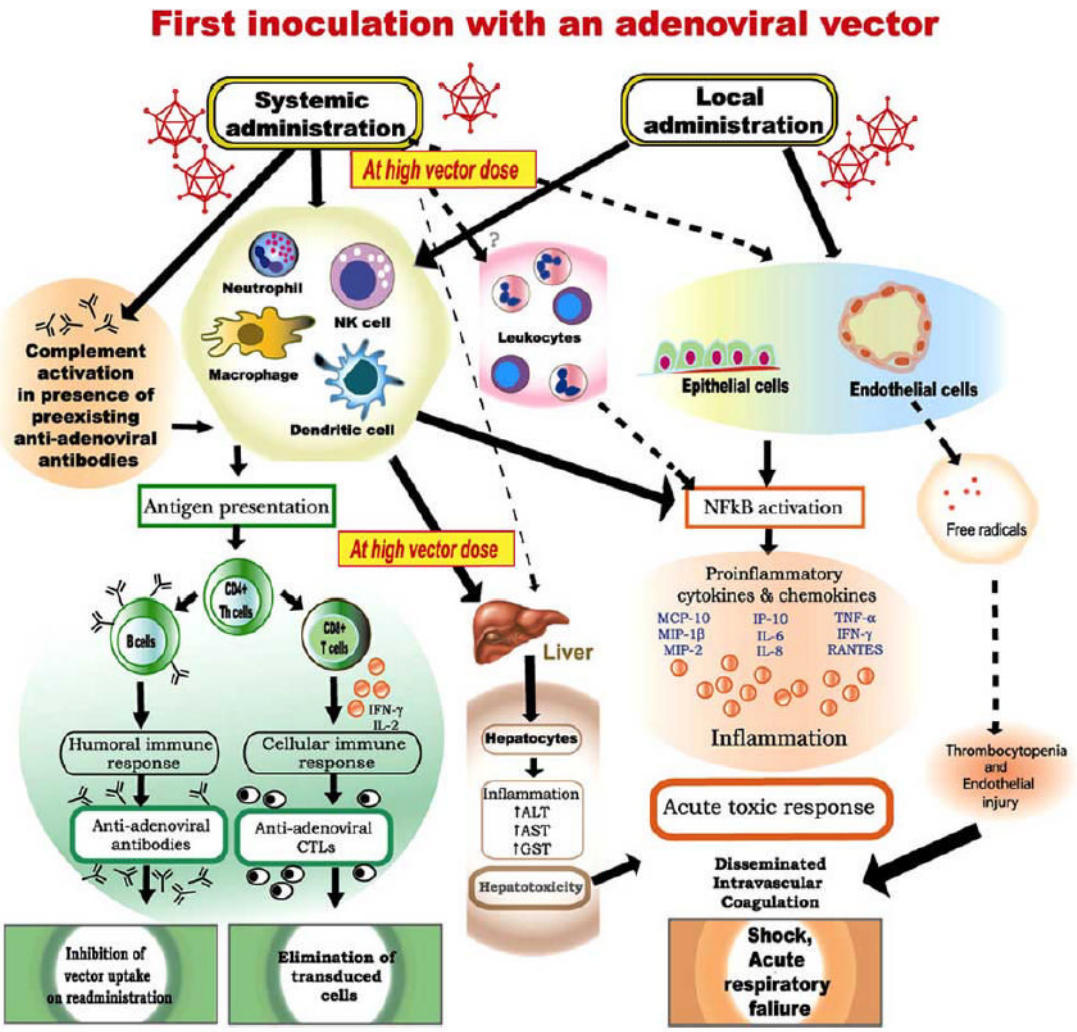

Fig. (2).

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.

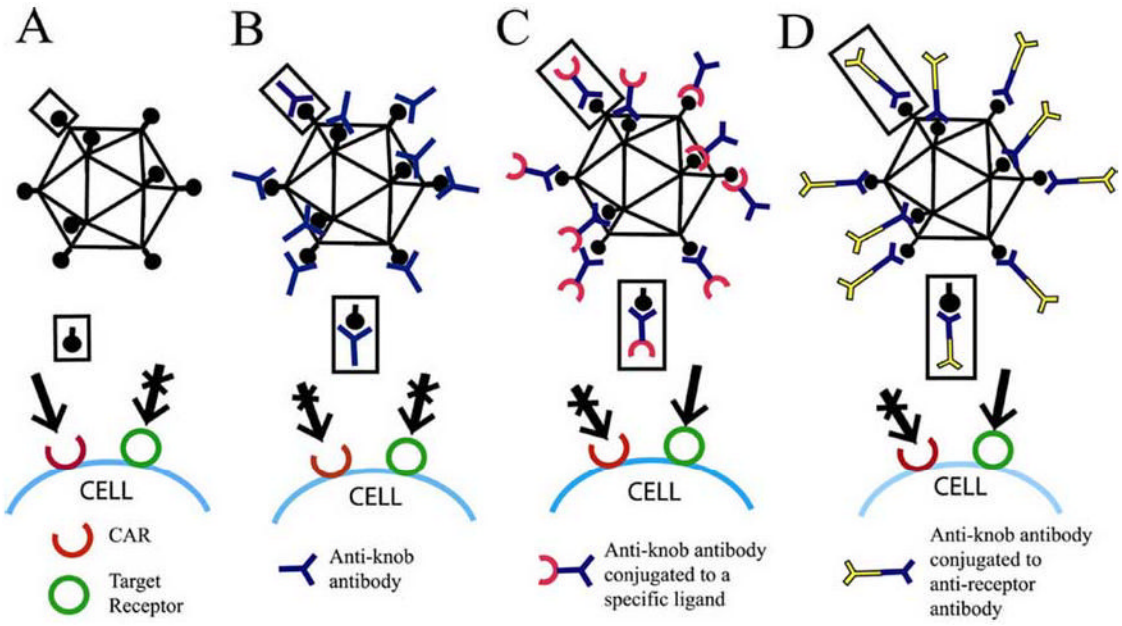

Fig. (3).

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 anti-receptor antibody to anti-knob antibody would target the Ad vector to the specific receptor on the cell surface.