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Review

Development of nonhuman adenoviruses as vaccine vectors

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Abstract

Human adenoviral (HAd) vectors have demonstrated great potential as vaccine vectors. Preclinical and clinical studies have demonstrated the feasibility of vector design, robust antigen expression and protective immunity using this system. However, clinical use of adenoviral vectors for vaccine purposes is anticipated to be limited by vector immunity that is either preexisting or develops rapidly following the first inoculation with adenoviral vectors. Vector immunity inactivates the vector particles and rapidly removes the transduced cells, thereby limiting the duration of transgene expression. Due to strong vector immunity, subsequent use of the same vector is usually less efficient. In order to circumvent this limitation, nonhuman adenoviral vectors have been proposed as alternative vectors. In addition to eluding HAd immunity, these vectors possess most of the attractive features of HAd vectors. Several replication-competent or replication-defective nonhuman adenoviral vectors have been developed and investigated for their potential as vaccine-delivery vectors. Here, we review recent advances in the design and characterization of various nonhuman adenoviral vectors, and discuss their potential applications for human and animal vaccination. © 2005 Elsevier Ltd. All rights reserved.

Keywords: Adenoviral vectors; Nonhuman adenoviral vectors; Adenoviral vector-based vaccine; Adenoviral vector immunity

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1. Introduction

Advancements in recombinant DNA technology, genomics and immunology have greatly impacted the process of vaccine development. Genetic manipulation of microbes to adapt them as vectors for gene delivery offers a unique opportunity for vaccine design. As more effective, novel and safe vaccines are sought for existing, emerging and reemerging diseases, viral and bacterial vectors are becoming an important immuno-prophylactic tool in the arsenal of modern medicine. The advantages of vectored vaccines include efficient antigen presentation and induction of both humoral and cell-mediated immunity. Among various vaccine vectors, adenoviral (Ad) vectors are being extensively investigated. Since the first use of Ad expression vectors in the early 1980s, Ad vectors have shown great promise as vaccine vectors [1–4]. In addition, Ad vectors are being extensively explored for their applications in gene therapy [5]. Ad vectors are also becoming a therapeutic tool for cancer therapy, by virtue of their own oncolytic nature. Several preclinical and clinical trials have demonstrated therapeutic potential of Ad vectors in the treatment of a variety of cancers [6–9]. Ad vectors are also being explored for genetic immunotherapy of tumors, as a clinical modality in conjunction with conventional chemo- and radiation-therapy [10].

1.1. Advantages of adenoviral (Ad) vectors

Ad vectors offer several advantages as vaccine vectors and meet the most important criteria of an ideal vaccine vector in terms of efficacy, safety and stability [4]. Ad can infect a broad range of both actively dividing as well as post-mitotic quiescent mammalian cells. Transgene expression with Ad vectors is usually robust and can be further enhanced by driving gene expression with strong heterologous promoters. Generation of various Ad vectors is fairly straightforward and the vectors can be easily grown to large scale in tissue culture. Since Ad vectors are replication-defective, there is no serious threat of horizontal transmission. The vector genome does not integrate into the host chromosome and mostly remains epichromosomal, thus making their use safe without a potential risk of insertional mutagenesis. Ad vectors induce strong immunity when administered via parenteral (subcutaneous, intravenous, intramuscular or intraperitoneal) or mucosal (oral or intranasal) routes. The latter is often desired as most infections occur at mucosal surfaces. Furthermore, Ad vectors appear to be safe for use in humans as human Ad (HAd) serotypes 4 and 7 have been used as live oral vaccines in military recruits in the USA for several decades without any known adverse effects [11].

Although the mechanism of immune response against foreign antigens expressed by Ad vectors is not fully understood, it is thought to involve both humoral and cellular arms of the immune system [4]. Following Ad vector administration, foreign gene product-specific antibodies are elicited in var-

ious experimental animal models [3,12]. These antibodies are primarily of the IgG2a isotype, indicating the predominance of Th1-type help. In addition, specific CD8+T cells also develop [13,14]. Ad vectors also induce a strong innate immune response [15] that presumably potentiates the subsequent humoral and cellular immune responses. Furthermore, transduction of antigen presenting cells [16] by Ad vectors facilitates antigen presentation and prolongs the duration of immune response.

1.2. Current status of Ad vector vaccines

Most vaccine studies using Ad vectors have been carried out with HAd serotype 5 (HAd5) as this expression system has been extensively used as a delivery vehicle in the majority of Ad vector-based gene therapy trials [17]. HAd5 is the best characterized of all adenoviruses. Several recent reviews have focused on the prospects of HAd5 vectors for vaccination [4,18,19]. A number of preclinical and clinical trials using HAd5 vectors as a vaccine are currently in progress [20–27]. The notable among them are: (i) an HAd5 vector vaccine against Ebola virus (EV) that elicited protective immunity against challenge with EV in primates; (ii) an HAd5 vector vaccine expressing human immunodeficiency virus (HIV)-1 env gene that induced protective immunity against HIV in rhesus monkeys as well as in baboons, and neutralizing antibodies in monkeys; (iii) an HAd5 vector that expressed a protective antigen of anthrax and showed effective protection against anthrax challenge; and (iv) HAd5 vectors expressing severe acute respiratory syndrome (SARS) corona virus protein that elicited a specific immune response in rhesus macaques. Ad vectors can also be used for passive immunotherapy as exemplified by a recent study [25], demonstrating the production of a single-chain antibody directed against a protective antigen of *Bacillus anthracis*.

1.3. Importance of vector immunity in immunization

Despite the above-mentioned advantages, clinical use of Ad vectors is severely hampered by the widely prevalent pre-existing immunity against HAd5 in the majority of the human population [28,29]. The efficacy of HAd5 vectors is expected to be highly variable depending on the levels of preexisting vector immunity. Since Ad vectors are highly immunogenic, their first use also leads to development of strong humoral and cellular immunity [30], which blocks subsequent use of the vector. This is a potential problem in vaccination strategies that require repeated administration. Suppressing effects of anti-HAd5 immunity are attributed to strongly neutralizing antibodies directed against viral capsid components [31,32] and HAd5-specific CD8+ T cells [33]. Neutralizing antibodies lead to inactivation of free Ad vector, thus blocking transduction of the target cell, a prerequisite for antigen expression (Fig. 1). Neutralizing antibodies drastically reduce uptake of the Ad vector by cells, leading to poor transgene expression resulting in blunting of the transgene-specific immune

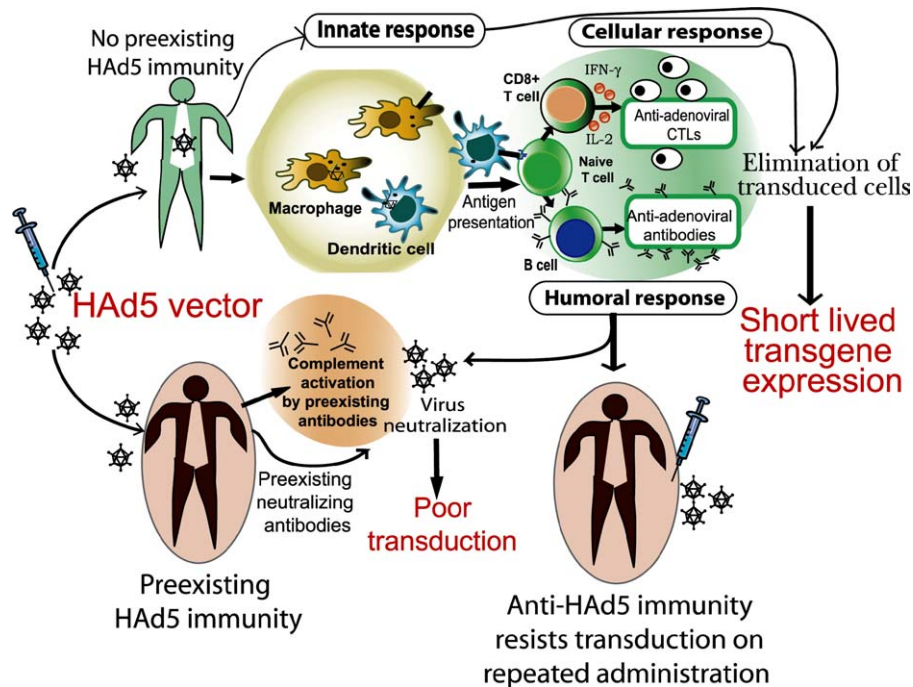


Fig. 1. Consequences of Ad vector immunity. Preexisting HAd immunity inhibits initial transduction with HAd vectors, and thus impedes the first use of many HAd vectors. In individuals with no preexisting HAd immunity, the first use of HAd vector would induce strong innate, cellular and humoral immune responses. The cellular immunity eliminates the majority of the transduced cells while the humoral immunity blocks subsequent infection with the same HAd vector.

response. Indeed, several studies have shown a drastic reduction in gene-transfer potential of HAd5 vectors in preimmune animal models [30,34–36]. Specific CD8+ T-lymphocytes will eliminate the vector transduced cells and thus shorten the duration of antigen expression. In case of the individuals that are not pre-exposed to HAd5, the first use of HAd5 vectors will lead to development of strong anti-HAd5 immunity that will make their subsequent use less effective (Fig. 1). This is a limitation of most of the live virus vector vaccines as many vaccine applications warrant more than one dose/booster of the vaccine in order to elicit protective immunity.

Preexisting HAd5 immunity drastically reduces the immunogenicity of HAd5 vector vaccines in animals [24,30,37] as well as in human beings [18]. Furthermore, since the level of anti-HAd5 neutralizing antibodies varies widely in the human population, immune responses following vaccination with HAd5 vectors will be highly variable and largely unpredictable [19]. Therefore, a preliminary screening of target individuals would be necessary prior to using HAd5 vectors.

In order to circumvent the preexisting HAd5 immunity, several approaches are under investigation. These include priming with recombinant DNA prior to boosting with a HAd5 vector [37,38], mucosal delivery of HAd5 vectors [39], microencapsulation of HAd5 vectors in inert polymers [40,41], engineering fiber- or hexon-chimeric HAd5 vectors [42–44], use of Ad vectors based on rare HAd serotypes [28,45], or use of vectors derived from nonhuman Ad types

[46–49]. The focus of this review is on nonhuman Ad vectors and this will be discussed in the following sections.

2. Nonhuman adenoviruses

Adenoviruses have been isolated from a variety of animal species and birds [50]. Based on their genome organization, serological properties and growth in cell culture, these viruses have been grouped into four distinct genera under the family *Adenoviridae* [51]. Most mammalian adenoviruses, including all known HAd serotypes belong to the genus *Mastadenovirus*. Adenoviruses isolated from birds are classified under the genus *Aviadenovirus*. The genus *Atadenovirus* consists of unique viruses isolated from ovine, bovine, deer, possum as well as some bird isolates. These viruses are distinct from the genus *Mastadenovirus* as they lack a distinct early region 1 (E1) transcriptional unit and have very high AT (adenine and thymidine)-rich genomes. Most recently, a fourth genus *Siadenovirus* was proposed that includes viruses isolated from frog, other invertebrates and the virus causing turkey hemorrhagic enteritis [50]. Like most HAd, the majority of nonhuman Ad, chosen for vector design do not cause significant clinical or pathological signs in their natural host species. Since Ad infections are widespread, the majority of the adult population of the host species is seropositive to the most prevalent Ad serotypes.

The precedent for using nonhuman Ad as expression vectors primarily came from their no or low pathogenicity

Table 1
Characteristics of various nonhuman adenoviruses being developed as vaccine vectors

Nonhuman adenovirus	Year of isolation	Genome size (bp) (Genbank accession no.)	Site/s for transgene insertion	Currently used cell line/s for vector generation	Examples of vectored vaccine [references]
Bovine adenovirus serotype 3 (BAd3)	1965	34,446 (AF030154)	E1A region	HAD5 E1-transformed fetal bovine retinal cells (VIDOR2 and FBRT HE1) BAD3 E1-complementing FBK 34 cells Bovine-human hybrid cells (BHH3 and BHH8)	Bovine herpesvirus gD [67]
			E3 region		Bovine virus diarrhea virus E2 glycoprotein [69]
Canine adenovirus serotype 2 (CAd2)	1962	31,323 (U77082)	E1A/B region	CAD2 E1A/B-transformed DK cells	Canine distemper virus HA of F antigen [79]
			E3 region		
Chimpanzee adenovirus (SAd25: C68 isolate)	1971	36,521 (AF394196)	E1A/B region	293 cells Other HAd5 E1-transformed cell lines	HIV-1 gag [14,86] Rabies virus glycoprotein [84]
Ovine adenovirus serotype 7 (OAd7)	1994	29,576 (U40839)	Site I- pVIII and fiber intergenic region Site II- unique <i>SalI</i> site within ORF RH2 Site III- region between the right end and the putative E4 transcription units	Sheep fetal lung (CSL503) cells Ovine fetal skin fibroblast producer cell line (HVO156)	Hepatitis C virus NS3 [96] <i>Taenia ovis</i> 45 W antigen [98]
Porcine adenovirus serotype 3 (PAd3)	1967	34,094 (AF083132)	E1 A region	HAD5 E1-transformed porcine fetal retinal cells (FPRT HE1-5 and VIDO R1)	Transmissible gastroenteritis virus S protein [104]
			E3 region		Classical swine fever virus gp 55 [106]
			Region between E4 promoter and the right ITR		
Fowl adenovirus serotype 1, CELO (FAd1)	1971	43,804 (U46933)	Three ORFs adjacent to the right end of the genome Region between 938 and 2900 (with trans-complementation)	Leghorn male hepatoma (LMH) cells	Infectious bursal disease virus (IBDV) VP2 [128]

for their natural hosts and similarity of their structural and genomic organization with that of HAd5 in the case of the *Mastadenovirus* species. Various nonhuman Ad that are currently under investigation as gene expression vectors include bovine Ad serotype 3 (BAd3), canine Ad serotype 2 (CAd2), chimpanzee Ad serotype 1 (for example; simian adenovirus serotype 25, SAd25), ovine Ad serotype 7 (OAd7), porcine Ad serotypes 3 and 5 (PAd3, PAd5) and fowl Ad serotype 1, 8, 9 and 10 (FAd1, 8, 9, 10) (Table 1).

With greater understanding of the biology of these non-human Ad and availability of suitable systems for vector rescue and propagation, replication-competent as well as replication-defective vectors have been constructed. Multiple sites for foreign gene insertion have been identified in the genomes of various nonhuman Ad (Fig. 2). In general, a two-plasmid strategy for homologous recombination in bacteria to generate the full-length recombinant Ad genome followed by transfection of a suitable EI-complementing cell line (Fig. 3) has been the mainstay of nonhuman Ad vector construction [52,53]. Chimpanzee Ad vectors can be rescued in EI-complementing cell lines of human origin following co-transfection of a shuttle plasmid and the wild-type genomic DNA [46]. For OAd7, a recombination strategy using a cosmid has also been used, in addition to the homologous recombination in bacteria. FAd vectors were developed by co-transfection of a shuttle plasmid and genomic DNA in a permissive cell line (Fig. 3).

Nonhuman Ad vectors have been characterized *in vivo*. Absence of virus neutralizing antibodies in human sera suggests that these viruses are not prevalent in the human population. Further, preexisting HAd5 antibodies in the sera

of human or experimental animals did not cross-neutralize most nonhuman Ad [36,48,49]. In addition, these vectors efficiently transduce several human and nonhuman cells [46,48,54–56], indicating their usefulness as alternative vectors for human as well as veterinary use. Below, we present a review of recent advances with each of these nonhuman Ad vectors.

2.1. Bovine Ad vectors

Ten Ad serotypes (1–10) have been isolated from cattle. These are classified either under the genus *Mastadenovirus* or *Atadenovirus* based on their genome organization and ability to grow in cell culture. Bovine Ad serotype 3 (BAd3) is a *Mastadenovirus* isolated from the conjunctiva of a healthy cow [57]. Complete nucleotide sequence and genomic map of BAd3 revealed a high level of similarity with HAd5 [58]. The E3 region of BAd3 is smaller and less complex than the E3 of HAd5 [58], and is not essential for virus replication in tissue culture [59]. A recent study compared the influence of various promoters for transgene expression in the E3 region of BAd3 [60]. The murine cytomegalovirus (MCMV) immediate early promoter was more efficient for foreign gene expression in the E3-parallel orientation than other exogenous promoters such as phosphoglycerate kinase (PGK) or human cytomegalovirus (HCMV) promoters. Development of EI-complementing cell lines of bovine origin was an important requirement in order to generate replication-defective BAd3 vectors since the HAd5 EI-expressing human 293 cell line [61] did not support BAd3 replication [59]. The E1 gene products of BAd3 were shown to complement E1A

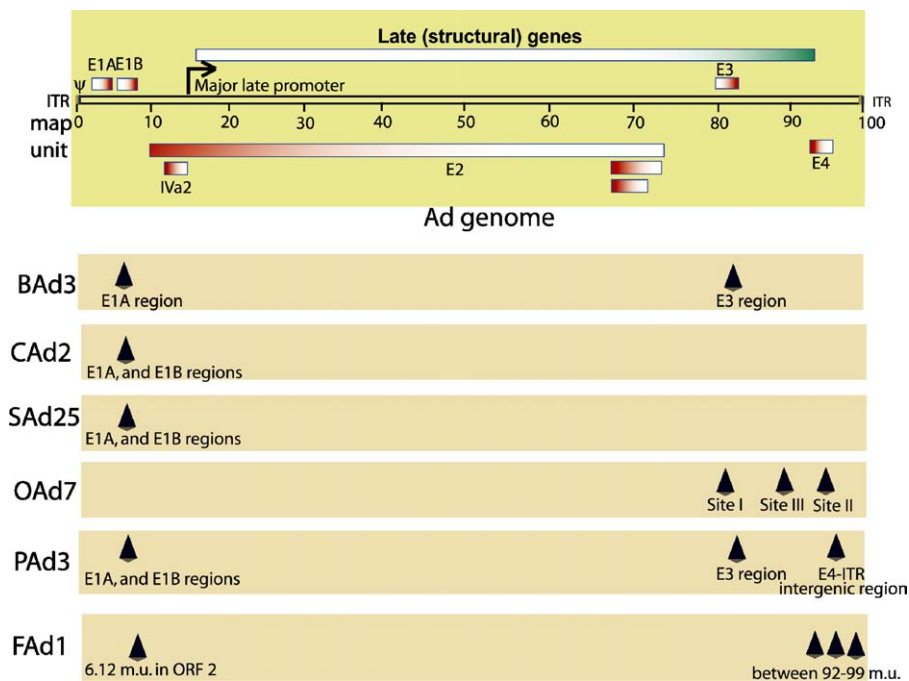


Fig. 2. Sites of foreign gene insertion in the genome of various nonhuman Ad vectors. Genome maps are drawn with respect to the genome map of type C HAd (shown at the top). ITR, inverted terminal repeat; Ψ , Ad packaging signal; E (early region gene); IVa2, IVa2 gene; ORF, open reading frame; m.u., map unit.

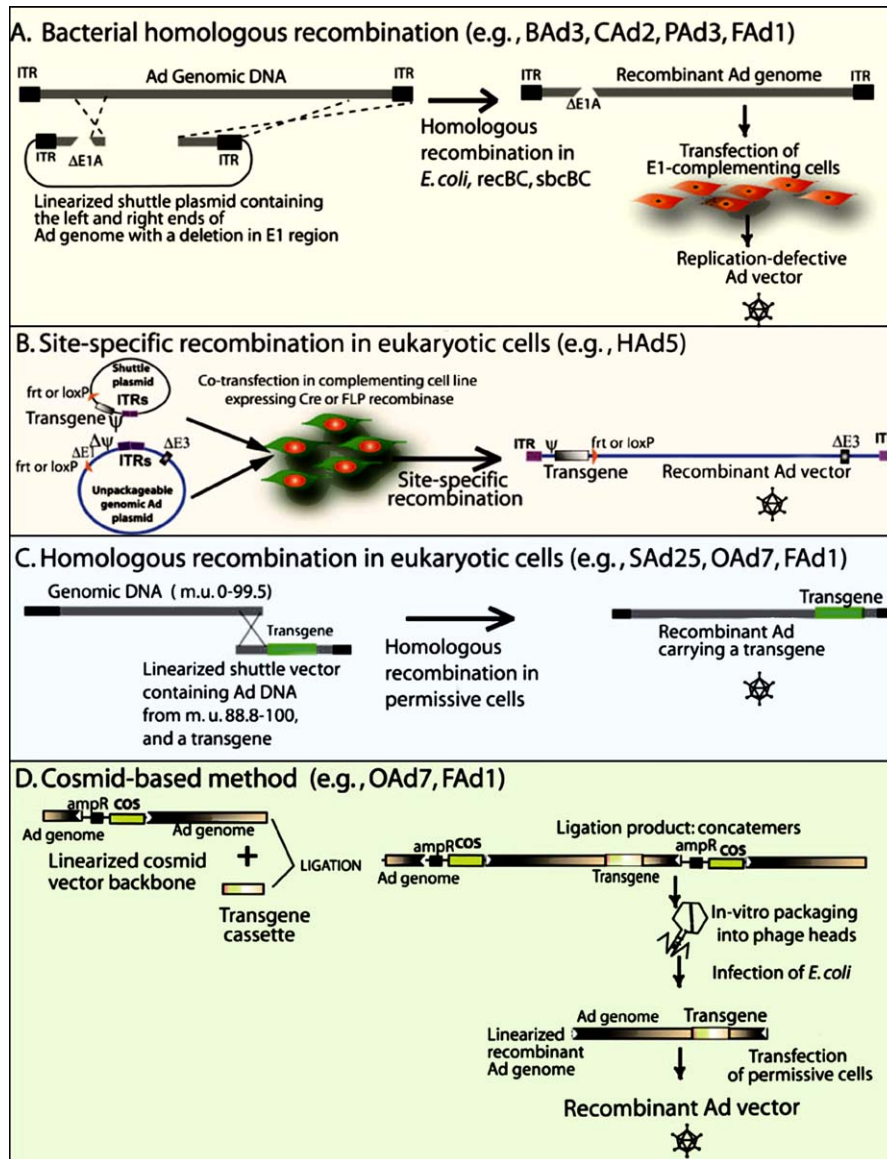


Fig. 3. Strategies for construction of nonhuman Ad vectors. ITR, inverted terminal repeat; $\Delta E1A$, deletion in early region 1A; $\Delta E3$, deletion in early region 3; Ψ , Ad packaging signal; m.u., map unit; frt, FLP recombinase target; loxP, Cre recombinase recognition site; amp^r, ampicillin resistance gene; COS, cohesive sites.

function of HAAd5 as demonstrated by replication of an E1-deleted HAAd5 vector in BAAd3 infected MDBK cells [62]. This trans-complementation, based on structural and functional homologies in the E1 genes of HAAd5 and BAAd3, led to the generation of complementing cell lines by transforming cells of bovine origin with HAAd5 E1 [63,64].

Subsequently, replication-defective BAAd3 with deletion in E1A and/or E3 were constructed by homologous recombination in *E. coli* BJ5183 [53] and rescued by transfecting E1-complementing bovine retina cells (VIDO R2, [63]; FBRT HE1 [56,64]) (Fig. 3). In addition, bovine cell lines expressing E1 proteins of BAAd3 have also been developed [64]. More recently, BAAd3 genome sequences involved in the virus packaging have been identified. This may be helpful in designing novel vectors with increased carrying capacity on the prece-

dence of helper-dependent 'gutless' HAAd5 vectors [65]. The ability to incorporate a heterologous protein in the C-terminus of minor capsid protein pIX protein of BAAd3 [66] offers a unique opportunity to insert specific ligands in the viral capsid to improve or alter transduction by BAAd3 vectors.

A number of studies have demonstrated the feasibility of generating a strong immune response against foreign gene products expressed by BAAd3 vectors. Mucosal immunization of calves with a replication-competent BAAd3 vector carrying bovine herpesvirus type 1 (BHV-1) glycoprotein gD in the E3 region induced neutralizing antibodies that conferred protection against BHV-1 challenge [67]. Comparison of replication-defective and replication-competent BAAd3 vectors expressing BHV-1 gD showed a lower immune response was elicited with the replication-defective vector

[68]. It is important to note that these vectors carried the foreign gene under the adenoviral major late promoter. Potentially, the problem of a lower immune response with a replication-defective vector could be overcome by driving the transgene expression with strong promoters, such as the HCMV or the simian virus 40 (SV40) early promoter. Another study investigated the feasibility of generating an immune response against bovine viral diarrhea virus (BVDV) glycoprotein E2 in cotton rats using an E3-deleted BAd3 vector [69]. BVDV E2-specific IgA and IgG responses were elicited following intranasal immunization with the vector.

Several studies examined preexisting immunity against BAd3 and its role in the subsequent use of BAd3 vectors in the cattle population [67,70]. Replication-competent BAd3 vectors could induce protective immune responses in calves that had pre-existing BAd3-specific antibodies. BAd3 vectors could also circumvent preexisting HAd5 immunity in a mouse model [36]. We recently reported that BAd3 is not prevalent in the human population, and that preexisting HAd immunity does not cross-neutralize BAd3 [56]. These studies demonstrated the potential of BAd3 vectors for gene transfer in humans even in the presence of HAd neutralizing antibodies. To further explore this possibility, it was important that BAd3 vectors efficiently transduce human cells. The wild-type BAd3 could infect but did not grow in human cell lines [54,59]. Replication-defective BAd3 vectors efficiently express foreign gene/s in a variety of human as well nonhuman cells in culture [54,56,63]. BAd3 entry into various cell types appears to be CAR- as well as integrin-independent [71]. In addition, tropism of BAd3 could be altered by switching the viral fiber knob with the HAd5 fiber knob [72], thus indicating the involvement of receptor/s distinct from primary receptors of HAd5 in BAd3 internalization [71].

2.2. Canine Ad vectors

Two serotypes of canine Ad (CAAd) have been isolated. Both CAAd1 and CAAd2 have been associated with mild upper respiratory tract infections in dogs. Effective live-modified vaccines are available for routine vaccination of dogs against both CAAd1 and CAAd2, with an excellent safety record. The complete genomic sequence of CAAd1 is known [73]. An E3-deleted CAAd vector expressing the capsid protein of canine parvovirus was generated by homologous recombination in bacteria [74]. CAAd2 is well characterized, and has been utilized for vector development. The CAAd2 genome carries an E1-transcription region similar to HAd5. Since E1-deleted CAAd2 vectors did not replicate in human E1-complementing cell lines, an E1-complementing canine kidney cell line (DK/E1-28) was developed that expressed CAAd2 E1 proteins [52,75]. Homologous recombination following co-transformation of a shuttle plasmid and CAAd genomic DNA in *E. coli* BJ5183 was adapted for generating recombinant infectious clones (Fig. 3). A conditionally replicating

CAAd2 vector, the first oncolytic vector based on a nonhuman Ad, was successfully tested for its therapeutic application in canine osteosarcoma [76]. With the availability of the information regarding the packaging sequence of CAAd2 [77], a system for generating helper-dependent vectors (with majority of the viral genome deleted) has been developed [78].

Based on the experience with commonly used live CAAd vaccines, it was observed that these CAAd vaccines were efficacious for the vaccination of dogs not only by the parenteral route but also by the mucosal route. Replication-competent CAAd vectors were expected to be useful in providing protective immunity in dogs and wild animals against a variety of pathogens including rabies. A replication-competent CAAd2 vector expressing canine distemper virus (CDV) hemagglutinin or fusion protein in the E3 region under the control of a truncated HCMV promoter, resulted in significant seroconversion and protective immunity [79]. Intranasal vaccination with this vector induced significant levels of CDV-specific immunity in seronegative puppies but a poor immune response was elicited in the puppies preexposed to CAAd2. The inability of the CAAd2 vector vaccine to elicit a CDV-specific serological response following intranasal inoculation was attributed to anti-CAAd2 mucosal immunity in preexposed animals.

Like most other Ad, CAAd are also prevalent in their natural hosts. Importantly, however, neutralizing antibodies against CAAd2 are not prevalent in humans [52]. CAAd2 vectors can efficiently transduce a variety of human cells [54,78] but do not grow in these cells. Although CAAd2 was found to interact with CAR [80], its tropism is distinct from HAd5 as indicated by preferential transduction of nervous tissue [78,81], and novel tropism of a chimeric HAd5 vector carrying the CAAd2 fiber [47]. This feature of CAAd2 should be helpful in designing alternate vectors for the targeted gene delivery.

2.3. Chimpanzee Ad vectors

Nine serotypes of chimpanzee Ad are currently known, which are classified as simian Ad (SAd) under *Mastadenovirus* genus [50]. The complete nucleotide sequences of SAd25 (also referred as AdC68 or Pan 6), as well as SAd22, SAd23 and SAd24 are now available [46,82,83]. SAd25 is grouped under HAd subgroup E based on its sequence homology and genome organization. These viruses do not cause any apparent clinical symptoms in chimpanzees. Unlike other nonhuman Ad, which were initially developed as vaccine vectors for their natural hosts, interest towards developing gene delivery vectors based on SAd has gained impetus primarily because of their ability to circumvent preexisting immunity to HAd vectors [13,46,84].

Because of their close homology with HAd, trans-complementation of E1 function of SAd viruses by HAd5 E1 was anticipated. The first E1-deleted replication-defective SAd25 recombinant carrying the enhanced green fluorescent

protein (EGFP) as a reporter gene was rescued by homologous recombination in the human EI-complementing 293 cell line [46] (Fig. 3). Rescue of recombinant SAd vectors in cell lines that complement HAd5 EI is not associated with any potential risk of generating replication-competent Ad vectors [46,84].

With continued efforts towards developing an efficacious vaccine against HIV, interest grew for an HAd vector vaccine against HIV-1 that has shown great promise in animal models [23,24,85]. Here again, the preexisting immunity against HAd5 in the human population may dampen the initial preclinical promise as these vaccines enter clinical trials. SAd25-based HIV-1 vaccines are, therefore, being investigated for their efficacy, safety and potential as an alternative to HAd5 vector vaccines [13,14,86]. An EI-deleted SAd25 vector expressing the truncated-form of the HIV-1 gag antigen induced a gag-specific CD8⁺ T cell response following oral administration in a mouse model [14]. The T cell response could be augmented with a systemic-booster immunization. Immunization using HAd5 and SAd25 vectors expressing the HIV-1 gag antigen induced strong, antigen-specific cellular as well as humoral responses in mice as well as monkeys [86].

Recently, molecular clones of three SAd serotypes, SAd-22, -23 and -24 (also referred to as Pan-5, -6 and -7, respectively) were developed by deleting their EI sequences [83]. Similar to SAd25, EI-functions of these SAd serotypes were also complemented by HAd5 EI, and replication-defective vectors could propagate in human 293 cells.

Various chimpanzee Ad, including SAd25, are not naturally prevalent in the human population [46,83]. In a panel of human, chimpanzee, or rhesus monkey serum samples, 80% of samples from the chimpanzee had neutralizing antibodies against SAd25 while only 2% of human or rhesus monkey samples had SAd25-neutralizing antibodies. Preexisting immunity against several common serotypes of HAd including HAd5, did not reduce the antibody response to the transgene expressed by an SAd25 vector in a mouse model [84]. Preexposure of mice to replication-defective or replication-competent HAd5 blunted the antibody response following inoculation with a HAd5 vector but did not affect after immunization with a SAd25 vector [84,87]. Similar observations were made for other SAd serotypes (SAd22, SAd23 and SAd24) [83]. Interestingly, antibodies against SAd22, SAd24, or SAd25 cross-neutralized each other but did not neutralize SAd23. SAd25 as well as SAd22, SAd23 and SAd24 efficiently transduced human HeLa and A549 cells [83]. Since various SAd serotypes do not cross-neutralize each other, these can be used for readministration of the immunogen.

Chimpanzee Ad vectors efficiently transduced various human cells in culture [46,83]. The transduction profile of these vectors was comparable to that of HAd5. It has been shown that SAd25 is internalized via the CAR receptor [88]. Ongoing research will provide further insight on their safety and efficacy for human vaccination.

2.4. Ovine Ad vectors

There are seven distinct serotypes of Ad isolated from sheep. These are referred to as ovine Ad serotypes 1–7 (OAd1–7). Serotypes 1–6 are assigned to the genus *Mastadenovirus*. Serotype 7 (OAd7, isolate 287 or AdC68) is a non-pathogenic Ad of sheep. It has a unique genome organization distinct from that of genus *Mastadenovirus* or *Aviadenovirus*. The complete nucleotide sequence of OAd7 is now available. The OAd7 genome is characterized by a high AT content and the lack of a clearly distinguishable EI region [89]. Owing to these differences, OAd7 along with Ad isolates from deer, possum, duck, snake, etc. is now classified under a new genus *Atadenovirus* [90]. Recombinant OAd7 vectors can be constructed and rescued by homologous recombination in *E. coli* followed by transfection of a permissive ovine fetal lung cell line (CSL503) [91] (Fig. 3). Another, more efficient cosmid-based strategy for vector generation following vector rescue in an ovine fetal skin fibroblastic cell line has also been developed [92]. Three dispensable, nonessential regions of the viral genome, where foreign genes can be inserted, have been delineated [93]. These are referred as the sites I, II and III (Fig. 2). The site I is located between the pVIII and fiber genes; the site II is located towards the right end of the viral genome [94]; and the site III is located between the E4 region and right end transcription unit [95]. Recombinant OAd7 vectors carrying transgene inserts at the site III are most stable making it the most preferred region for foreign gene insertion. The biology and potential gene delivery applications of OAd7 have recently been reviewed [93].

A number of recombinant OAd7 vectors expressing foreign genes encoding immunogenic proteins have been developed. An OAd7 recombinant expressing hepatitis C virus NS3 protein elicited a strong and sustained antigen specific T-cell response in mice [96]. High levels of transgene were expressed following vector administration by intravenous route [49]. Vector biodistribution was similar in the spleen, heart, kidney and the liver while the lung was infected to a lower degree. Similarly, high serum levels of the transgene product were produced following intramuscular administration of an OAd7 vectors expressing human alpha 1-antitrypsin gene in mice [97]. An OAd7 vector expressing *Taenia ovis* antigen 45 W antigen inserted at site I under the control of the viral major late promoter elicited effective immunity against parasite challenge when used in combination with a DNA vaccine in a prime/boost approach [98].

Recombinant OAd7 vectors have also been used for gene therapy [99]. A recombinant vector expressing human α 1-antitrypsin (hAAT) gene under the control of the Rous sarcoma virus promoter was used for investigating gene transfer potential and in vivo distribution of the vector in immunocompetent as well as immunodeficient mouse models. The level and duration of hAAT gene expression were similar to those achieved with a HAd5 counterpart. However, the tissue-tropism of the OAd7 vector differed from that of HAd as the liver was not the dominant target. Transgene expres-

sion was stable in the immunodeficient mice implying the role of vector immunity in clearance of transduced cells. Importantly, efficient gene transfer with the OAd7 vector in mice immunized with HAd5 vectors and vice versa [49], thus underscoring the potential of OAd7 vectors for circumvention of HAd5 immunity. Other studies have investigated the usefulness of OAd7 vectors in gene-directed enzyme-prodrug therapy approach [99,100]. Systemic administration of a single dose of a recombinant OAd7 vector carrying the *E. coli* purine nucleoside phosphorylase (PNP) gene followed by the prodrug fludarabine phosphate resulted in significant suppression of prostate cancer progression in an immunocompetent mouse model [100].

A serological survey of human serum samples indicated that OAd7 neutralizing antibodies are not prevalent in humans [49]. Furthermore, HAd5 neutralizing antibodies do not cross neutralize OAd7 [49]. Anti-HAd5 antibodies present in experimental animals impeded gene transfer with HAd5 vectors but did not prevent gene transfer with OAd7 vectors, indicating the potential of these vectors for evading HAd5 immunity [49]. OAd7 vectors efficiently transduced a variety of human cells in culture. Importantly, OAd7 infection of several nonovine cells tested was abortive as progeny virus could not be detected despite some replication of viral DNA [101]. These observations have significant impact with respect to suitability and safety of the OAd7 vector system for future use in humans. Although receptors involved in OAd7 internalization are not known, OAd7 appears to use receptors distinct from those of HAd5. This is supported by distinct *in vivo* tropism and differential transduction profile of OAd7 and HAd5 [49,93], as well as altered tropism of a chimeric OAd7 vector carrying the fiber knob domain of HAd5 [102].

2.5. Porcine Ad vectors

Five serotypes of porcine Ad (PA1-5) are currently known to infect pigs [51]. PA3 is the most prevalent serotype, and was isolated from a healthy piglet. It is not associated with any clinical disease in pigs. PA3 is not known to naturally infect species other than porcine. Being a *Mastadenovirus*, PA3 shares structural and genomic organization with HAd5 [103]. The PA3 genome consists of distinct E1A, E1B, E2, E3 and E4 transcription regions. Replication-competent as well as replication-defective PA3 vectors have been developed as gene delivery vehicles [48,55,104]. The E3 region is dispensable for virus growth and has been used for inserting foreign genes [104].

The potential utility of PA3 for vaccine purpose was demonstrated before the vector construction was attempted. Oral vaccination of pigs with PA3 stimulated both systemic and mucosal virus neutralizing antibodies [105]. First use of a recombinant PA3 in pigs was carried out with a vector having no deletions and carrying the classical swine fever virus (CSFV) gp55 (E2) gene cassette in the right hand end of the viral genome [106]. This vector induced high levels of anti-gp55 antibodies following a single subcutaneous inoculation

in outbred pigs leading to complete protection from CSFV challenge. Subsequently, a prime-boost vaccination strategy involving vaccination of weaned and neonatal pigs first with a DNA plasmid carrying the CSFV gp55 and then with a recombinant PA3 expressing CSFV gp55 [107], conferred complete protection against experimental CSFV challenge in weaned pigs. A PA3 vector carrying the pseudorabies (Aujeszky's disease) virus (PRV) gD gene [104] in the E3 region yielded high levels of PRV-specific antibodies in vaccinated pigs [107]. Animals that received two doses of the vector showed improved antibody titers and increased protection in comparison to those given a single dose.

Although replication-competent PA3 vectors may be very useful for vaccine purposes in swine, their use in heterologous species including humans may not be favorable due to safety concerns. Therefore, an important requirement for potential application of PA3 vectors in species other than swine was to render them replication-defective. The E1 region of PA3 is homologous to the E1 region of HAd5. Like BA3 E1, PA3 E1 can also complement the E1 functions of HAd5. Reciprocally, HAd5 E1 can complement E1A functions of PA3 [48,108]. A number of E1-complementing cell lines were made by transforming porcine retinal cells with HAd5 E1 [48,55,104]. This trans-complementation facilitated generation of E1A-deleted PA3 vectors in porcine cell lines that express HAd5 E1 (Fig. 3).

As mentioned earlier, Ad are widely prevalent in their natural hosts. PA3 is the most prevalent serotype in pigs [109]. In a survey to determine the extent of preexisting PA3 immunity in Australia, 90% of pigs tested had significant titers of virus neutralizing antibodies. Consequently, concerns were expressed regarding the suitability of PA3 vectors for use in swine herds [110]. However, a study where pigs were preexposed to wild-type PA3 prior to vaccination with a recombinant PA3 vector expressing CSFV gp55, revealed that efficacy of the vector vaccine was not inhibited by the presence of PA3 specific neutralizing antibodies [111]. Thus, the PA3 vector still holds great potential for use in swine.

Importantly, we recently reported that PA3 is not prevalent in the human population as indicated by the lack of neutralizing antibodies in human serum samples [48]. HAd5 neutralizing antibodies in human serum did not cross neutralize PA3. It has been demonstrated that PA3 can efficiently overcome anti-HAd5 immunity in a mouse model [36]. PA3 efficiently transduced cells of human, murine, porcine or bovine origin, indicating its usefulness as a gene delivery vector [56]. Being highly species-specific, PA3 infects but does not replicate in the cells of canine, ovine, bovine and human origin [104]. Unlike HAd5, transduction by PA3 appears to be CAR- and integrin-independent [112], indicating its distinct tropism and potential application in designing novel targeted vectors.

PA5 is also being investigated as a potential gene expression vector. PA5 was originally isolated in Japan [113] and it does not cause any serious disease in pigs. Its complete

genomic sequence is now available [114]. Since the majority of the E3 region is not essential for virus replication, PA5 vectors carrying the 2.2 kb S gene fragment of swine transmissible gastroenteritis virus (TGEV) in the E3 region were constructed [115,116]. These vectors induced TGEV-specific systemic as well as mucosal immune responses following oral administration of pigs [115].

2.6. Fowl Ad vectors

Several Ad have been isolated from birds [117]. Most of these are included in the genus *Aviadenovirus* under three serological groups I, II and III [51]. Some other avian Ad (such as Egg Drop Syndrome 76 virus) are included in the new genus *Atadenovirus* [51], based on their distinct structural and genomic features. Group I *Aviadenovirus* includes fowl Ad (FAd) 1–12, and are mostly nonpathogenic or cause only mild infections. FAd1 (Chicken embryo lethal orphan, CELO) was isolated from chickens [118], and is not associated with any disease. Inoculation of 1-day-old specific pathogen-free (SPF) chickens with FAd1 via various routes induced anti-FAd1 antibodies 3–4 weeks post inoculation, and did not cause any adverse clinical or pathological effects. FAd1 is distinct from mammalian Ad as it possesses a larger genome (43804 bp) and two fibers rather than a single fiber at each vertex of the viral capsid. The complete nucleotide sequence of FAd1 is known and contains no recognizable regions corresponding to HAd5 E1A, E1B, E3 or E4 [119]. FAd1 E1 functions can not be complemented by HAd5 E1 [120]. However, FAd1 can transform mammalian cells [121], and induce tumors in hamsters [122]. The transforming genes of FAd1 are partially characterized. The unique FAd1 open reading frame 8 encodes an anti-apoptotic protein, GAM-1 (*Gallus anti morte protein 1*), which is an E1B 19K protein homologue [123], and interacts with retinoblastoma protein leading to E2F pathway of transcription activation [124]. Deletion of the GAM-1 gene renders the virus replication-defective, and the defect could be partially recovered by inducing a heat shock in the producer cell line [125].

Initially, FAd1-based vectors were constructed by using the classical approach of homologous recombination in bacteria to generate the recombinant Ad genome, followed by transfection of the permissive Leghorn male hepatoma (LMH) cells to rescue replication-competent vectors [126] (Fig. 3). A cosmid-based strategy has also been adopted for generating FAd1 recombinants [127]. Deletions up to 3.6 kb could be introduced between map units 91 and 99 in FAd1 genome that allow for foreign gene insertion up to 4 kb (Fig. 2). Immunization of chickens with a recombinant FAd1 vector expressing infectious bursal disease virus (IBDV) VP2 protein provided complete protection against virulent IBDV challenge [128]. The immunity induced by this vector was similar to that induced by the fowlpox virus vector, Marek's disease virus vector, or turkey herpesvirus vector, thus establishing utility of FAd1 as a vaccine vector. Recently, an FAd1 vector has also been examined for cancer

therapy in a mouse model of subcutaneous melanoma [129]. FAd1 efficiently transduced primary human dermal fibroblast, HepG2 and A549 cells with efficiency comparable to HAd5 vectors. Since FAd1 infects but does not replicate in a variety of human, porcine, equine and simian cell lines [126,127], FAd1-derived vectors may also be of potential application for vaccination of these non-avian species.

In addition to FAd1, FAd8, FAd9 and FAd10 have also been explored for developing expression vectors. Oral vaccination of 1-day-old broiler chickens with recombinant FAd8 expressing the S1 gene of infectious bronchitis virus (IBV) inserted at a 2.4 kb or 50 bp deletion site of the FAd8 genome elicited an IBV S1-specific antibody response [130]. A single vaccination was found to impart protective immunity against challenge with a homologous IBV. Another recombinant FAd8 vector expressing chicken interferon gamma was used for in vivo delivery of cytokine and tested for its ability to boost immunity to a coccidial parasite in chickens [131]. For FAd9, deletions in the long tandem repeat region 2 (TR-2) towards the right end of the viral genome were not lethal, and the insertion of a reporter gene was well tolerated [132]. These manipulations did not significantly alter the distribution, replication, and immune responses compared to the parent virus [133]. A recombinant FAd10 vector expressing IBDV VP2 was constructed by inserting the expression cassette in the right end of the genome between map units 90.8 and 100, upstream of the inverted terminal repeat [134]. Intravenous inoculation of this recombinant vector in 3-week-old birds resulted in protective immunity against IBDV. However, the vector instillation into the conjunctival sac (mucosal route) failed to elicit significant immunity against IBDV VP2, highlighting the importance of the route of administration in successful vaccination using FAd vectors.

3. Conclusions and future directions

Clinical use of HAd vectors is hampered by vector immunity that is prevalent in the majority of the human population, and also develops rapidly after first use of the vector. Nonhuman Ad are not prevalent in humans, and are not cross-neutralized by HAd neutralizing antibodies. Significant progress has been made in the understanding of the biology of these vectors. These vectors efficiently transduce human cells in culture and circumvent preexisting HAd immunity. An ever increasing number of preclinical and clinical studies demonstrating the utility of HAd vectors for therapeutic or prophylactic gene delivery have provided further momentum to the research efforts in the area of developing nonhuman Ad vectors. In addition, these nonhuman Ad vectors also present a platform for designing improved vaccines for veterinary use.

While, all nonhuman Ad vectors presented in this review have shown initial promise for gene transfer to human cells and evasion of HAd5 immunity, further investigation is needed before their use in humans could become a reality.

An important concern with nonhuman Ad vectors would be their safety for humans. Despite the fact that most nonhuman Ad vectors are replication-defective in human cells, and deletion of essential genes leads to blunting of viral replication, thorough examination of viral gene expression and viral genome persistence in a variety of human cells and in a nonhuman primate model will be necessary to ascertain safety of these vectors. Similarly, information regarding packaging sequences, receptor utilization and other determinants of tissue tropism, and in vivo persistence and state of the vector genome will determine the relative usefulness of these vectors. Although preliminary studies have shown the feasibility of sequential administration of various nonhuman Ad vectors, this aspect needs to be investigated further in suitable animal models. It will be important to investigate whether the humoral immune response against nonhuman Ad vectors cross-neutralizes other nonhuman Ad vectors. Such information will be useful for devising strategies for booster vaccination. Feasibility of construction of a 'minimal genome' Ad vector (such as gutless or gutted) by deleting the majority of viral genes in order to increase both the duration and expression of the target genes and the packaging capacity of all these nonhuman Ad vectors needs to be explored. Such vectors will be useful in developing vectors carrying multiple genes encoding antigens and/or immunomodulatory proteins. Careful assessment of optimal dose of these vectors, and any associated vector toxicity would also be required. For this purpose, development of appropriate laboratory animal models will be a logical next-step.

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