Novel replication-incompetent adenoviral B-group vectors: high vector stability and yield in PER.C6 cells

M. Havenga,[1] R. Vogels,[1] D. Zuijdgeest,[1] K. Radosevic,[1] S. Mueller,[2] M. Sieuwerts,[1] F. Weichold,[2] I. Damen,[1] J. Kaspers,[1] A. Lemckert,[1] M. van Meerendonk,[1] R. van der Vlugt,[1] L. Holterman,[1] D. Hone,[2] Y. Skeiky,[2] R. Mintardjo,[1] G. Gillissen,[1] D. Barouch,[3] J. Sadoff[2] and J. Goudsmit[1]

Correspondence
M. Havenga
m.havenga@crucell.com

[1]Crucell Holland BV, PO Box 2048, 2301 CA Leiden, The Netherlands

[2]AERAS Global TB Vaccine Foundation, 7500 Old Georgetown Road, Bethesda, MD 20814, USA

[3]Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, USA

Adenoviral vectors based on adenovirus type 35 (rAd35) have the advantage of low natural vector immunity and induce strong, insert-specific T- and B-cell responses, making them prime-candidate vaccine carriers. However, severe vector-genome instability of E1-deleted rAd35 vectors was observed, hampering universal use. The instability of E1-deleted rAd35 vector proved to be caused by low pIX expression induced by removal of the pIX promoter, which was located in the E1B region of B-group viruses. Reinsertion of a minimal pIX promoter resulted in stable vectors able to harbour large DNA inserts (>5 kb). In addition, it is shown that replacement of the E4-Orf6 region of Ad35 by the E4-Orf6 region of Ad5 resulted in successful propagation of an E1-deleted rAd35 vector on existing E1-complementing cell lines, such as PER.C6 cells. The ability to produce these carriers on PER.C6 contributes significantly to the scale of manufacturing of rAd35-based vaccines. Next, a stable rAd35 vaccine was generated carrying *Mycobacterium tuberculosis* antigens Ag85A, Ag85B and TB10.4. The antigens were fused directly, resulting in expression of a single polyprotein. This vaccine induced dose-dependent $CD4^+$ and $CD8^+$ T-cell responses against multiple antigens in mice. It is concluded that the described improvements to the rAd35 vector contribute significantly to the further development of rAd35 carriers for mass-vaccination programmes for diseases such as tuberculosis, AIDS and malaria.

Received 16 February 2006
Accepted 20 April 2006

INTRODUCTION

Mass vaccination for diseases such as AIDS, malaria and tuberculosis (TB) requires vaccine technology that can be produced on a large scale and that induces protective immunity. Protection against all three diseases is afforded primarily through strong $CD4^+$ and $CD8^+$ T-cell responses. Adenoviral vectors have been shown to induce such insert-specific cellular immune responses in diverse disease areas and animal models (Arribillaga *et al.*, 2002; Casimiro *et al.*, 2003; Jaiswal *et al.*, 2003; Pinto *et al.*, 2003; Shanley & Wu, 2003; Sullivan *et al.*, 2003; Tan *et al.*, 2003; Liu *et al.*, 2005; Phillpotts *et al.*, 2005; Seaman *et al.*, 2005; Wu *et al.*, 2005), as well as in humans (Shiver *et al.*, 2002). Also, the technology for manufacturing replication-incompetent adenoviral vectors is most matured, i.e. millions of vaccine dosages can be produced on appropriate cell lines, such as PER.C6 (Fallaux *et al.*, 1998). By far the most experience with adenoviral vectors has been obtained by using replication-incompetent human adenovirus serotype 5 (rAd5), particularly using human immunodeficiency virus- and simian immunodeficiency virus (SIV)-derived inserts (Shiver & Emini, 2004; Barouch & Nabel, 2005).

We have shown that the seroprevalence of antibodies against Ad5 is high in human populations in both the developed (Vogels *et al.*, 2003) and developing (Kostense *et al.*, 2004) worlds, and demonstrated that the presence of even low-level anti-Ad5 neutralizing antibodies suffices to blunt rAd5 vaccine potency completely (Lemckert *et al.*, 2005; Sumida *et al.*, 2005). To circumvent pre-existing anti-Ad5 immunity, we have generated vectors based on low-seroprevalence human adenovirus, i.e. rAd11 and rAd35 (Vogels *et al.*,

Published online ahead of print on 2 May 2006 as DOI 10.1099/vir.0.81956-0.

2003; Holterman *et al.*, 2004), and demonstrated that these vectors induce potent cellular immune responses in both naïve mice and in mice carrying high-titre anti-Ad5 neutralizing antibodies (Barouch *et al.*, 2004; Ophorst *et al.*, 2006). Thus, preclinical data suggest that rAd11 and rAd35 represent attractive vaccine carriers for the development of an AIDS, TB or malaria vaccine.

However, we observed that spontaneous deletion of transgenes larger than 2 kb occurred frequently in rAd35 vectors. Also, by using the novel, specifically engineered PER.C6/55K packaging cell line that complements for the E1 deficiency in rAd35 and, in addition, expresses Ad35-derived E1B-55k protein (Vogels *et al.*, 2003), a high variation within the virus particle (VP) : p.f.u. ratio was observed consistently. These findings hamper clinical applicability of rAd35, due to anticipated problems with vaccine stability and large-scale production. As we consider that an rAd35-based vaccine for TB, AIDS or malaria must be manufactured at least at a 10 000 l scale in order to obtain 100 million doses (10^{10} VP per dose), we deemed it pivotal to solve the problems of rAd35 genome instability and vaccine-production capacity.

METHODS

Adenoviral-vector plasmids and TB-S construct. To generate an rAd35 genome carrying Ad5-derived E4-Orf6 sequences, the Ad5 E4-Orf6 sequence was first cloned into a pBr plasmid containing Ad35 sequences (nt 18138 to right ITR: GenBank accession no. AY271307). This plasmid, called pBr.Ad35PRN, served as a template to amplify an Ad35 genome fragment of 1·8 kb (nt 30099–31880, containing a 3′ tail homologous to Ad5 sequence) by using primers E4-F1 (5′-AGAGGAACACATTCCCCC-3′) and E4-R2 (5′-GGGGAGAAAGGACTGTGTATTCTGTCAAATGG-3′). A second PCR was performed, using as template a cosmid clone containing Ad5 sequences from nt 3534 to the right ITR (pWE.Ad5.AflII-rITR; Vogels *et al.*, 2003; Holterman *et al.*, 2004) and primers E4-F3 (5′-TTTGACAGAATACACAGTCCTTTCTCCCCGGCTGG-3′) and E4-R4 (5′-ACAAAATACGAGAATGACTACGTCCGGCGTTCC-3′). This amplification reaction resulted in a 1·1 kb fragment corresponding to Ad5 sequence nt 32963–34077 (numbering as in GenBank accession no. M73260) and flanked by sequences homologous to the first (3′ end) and third (5′ end) PCR fragments. A third, 357 bp fragment corresponding to nt 32972–33329 in the Ad35 genome sequence and having a 5′-homologous tail to the second PCR fragment was obtained by using pBr.Ad35PRN as template in combination with primers E4-F5 (5′-GGACGTAGTCATTCTCGTATTTTGTATAGC-3′) and E4-R6 (5′-TCACCAACACAGTGGGGG-3′). The three PCR fragments were combined via assembly PCR using the two outer primers (E4-F5 and E4-R2). The resulting 2·8 kb DNA fragment was digested with *Mlu*I and *Nde*I and cloned in an *Mlu*I–*Nde*I-digested plasmid called pBr.Ad35.PRNΔE3. This gave rise to a plasmid called pBr.Ad35.PRNΔE3.5Orf6. Plasmid pBr.Ad35.PRNΔE3 is similar to pBr.Ad35.PRN, but carries a 2·6 kb deletion within the E3 region (corresponding to nt 27648–30320 of the Ad35 genome).

To create a new adaptor plasmid containing the pIX promoter, plasmid pAdapt35.IPI (Vogels *et al.*, 2003) was modified. Hereto, first an *Age*I restriction site located in the multiple cloning site behind the cytomegalovirus (CMV) promoter needed to be removed by partial *Age*I restriction, followed by filling in protruding ends with Klenow enzyme and religation. This plasmid was subsequently digested with *Bgl*II, blunted with Klenow and further digested with *Age*I. Ligation of the blunted *Age*I DNA fragment to a *Bsu*36I–*Age*I fragment corresponding to nt 3234–4251 in Ad35, in which the *Bsu*36I site was blunted with Klenow enzyme, resulted in the expected plasmid called pAdapt35.Bsu.

To generate an Ad35.E1B.Luc vector, a pBr plasmid containing Ad35 genome sequence (left ITR to nt 4669) was used in which the E1A sequences between *Sna*BI and *Hin*dIII (nt 452–1338 in Ad35) were replaced by an *Avr*II–*Bgl*II fragment (CMV-Luciferase-SV40 pA) derived from pAdapt35.Luc (Vogels *et al.*, 2003). This plasmid thus contains the E1B promoter and coding sequence in its native position relative to pIX.

To generate rAd35E1B.ΔpXI.Luc vector, the adaptor used to generate Ad35.E1B.Luc vector was further modified to carry a deletion in the pIX coding region (nt 3484–3805 in Ad35), thereby removing most of the pIX coding region.

The triple-antigen insert (TB-S), as well as single antigen-encoding DNAs, were obtained via gene synthesis of codon-optimized DNA sequences for expression in humans (Geneart GmbH) and contain the *Mycobacterium tuberculosis* antigens Ag85A (SwissProt accession no. P17944; aa 44–338), Ag85B (SwissProt accession no. P31952; aa 41–325) and TB10.4 (SwissProt accession no. O53693; full sequence). The three coding sequences are linked directly, thus without signal peptides or intervening sequences, in the order Ag85A–Ag85B–TB10.4. Sequences were cloned unidirectionally into the pAdap35.Bsu plasmids and vectors carrying Ad5-derived Orf6 were generated.

Cell culture and vector generation, purification and titration. Cell lines PER.C6, PER.C6/55K and A549 were cultured at 37 °C/10 % CO_2 in Dulbecco's modified Eagle's medium (Life Technologies Inc.) containing 10 % fetal bovine serum (Life Technologies Inc.) and further supplemented with 10 mM $MgCl_2$ (PER.C6 only). Standard replication-incompetent rAd35 vectors (carrying Ad35 E4-Orf6) were generated, manufactured on PER.C6/55K, purified and titrated as described previously (Vogels *et al.*, 2003; Holterman *et al.*, 2004). Recombinant Ad35 vectors carrying the Ad5 E4-Orf6 sequence were generated by co-transfection on PER.C6 cells of: (i) an adaptor plasmid based on pAdapt35.IPI (Vogels *et al.*, 2003) or pAdapt35.Bsu carrying a desired transgene cloned in the former E1 region (eGFP, SIVgag, TB-S); (ii) a pWE15-based cosmid containing Ad35 sequences (nt 3401–24649) called pWE.pIX-*Eco*RV; and (iii) plasmid pBr.Ad35.PRNΔE3.5Orf6. Prior to transfection into PER.C6 cells using Lipofectamine (Invitrogen) according to the manufacturer's instructions, plasmids were digested to liberate the adenoviral sequences from the plasmid backbones. Batches of rAd35.TB-S, single-antigen rAd35 vaccines, as well as all other rAd35 vectors were generated on PER.C6 in the case of the E4-Orf from Ad5 being located in the rAd35 backbone or on PER.C6/55K cells in the case of the E4-Orf6 of Ad35 region still being present. Homologous recombination between the shared sequences of the virus gave rise to full-length Ad35 genomes carrying the gene of interest. Viruses were plaque-purified twice and propagated on adherent cultures in 10–24 triple-layer flasks (Nunc). Purified stocks were obtained by standard two-step CsCl-gradient banding and the isolated virus was dialysed in three steps to a final formulation in PBS/5 % (w/v) sucrose. Virus concentrations were determined based on the optical density method described by Maizel *et al.* (1968) in the presence of 1 % (w/v) SDS. Infectivity was measured by plaque assay on PER.C6/55K for E1-deleted Ad35 viruses or by $TCID_{50}$ assay on 911 cells (Fallaux *et al.*, 1996) for rAd35 viruses carrying the Ad5 E4-Orf6 sequence. Determination of VP number in crude lysates was done by an HPLC method, essentially as described by Shabram *et al.* (1997).

Viral-genome analyses and thermostability assay. Viral DNA was isolated from crude lysate or purified vector batches by using a

GeneClean Spin kit (Bio101 Inc.) essentially according to the instructions provided. Viral DNA was used as a template to analyse the integrity of the transgene region by PCR amplification using a forward primer (#57) hybridizing to the 5′ end of the CMV promoter (5′-GTAGGTGTCAGCCTAGGTGGTC-3′) and a reverse primer (#342) hybridizing to the pIX coding sequence (5′-GGCGGGTTGAACGGGTCTTCCA-3′).

PCR amplification was performed with *Taq* DNA polymerase (2·5 U; Invitrogen) in 1× supplied buffer, 0·2 mM dNTPs, 2 mM $MgCl_2$, 0·6 mM each primer, 3 % DMSO and 5 µl viral DNA (or 50 ng control plasmid) in a volume of 50 µl. Mixtures were heated to 98 °C for 2 min and subjected to 30 cycles of 30 s at 95 °C, 30 s at 60 °C, 1 min kb^{-1} at 72 °C, followed by 8 min at 68 °C [ramping ($dT\ s^{-1}$), 2 °C s^{-1}].

To determine viral thermostability, A549 cells were seeded at 5×10^4 cells per well in 24-well plates 1 day before infection with a predetermined amount of vector. All vectors to be tested were first titrated on A549 cells to determine the volume of crude lysate that yielded approximately 80 % of the maximum luciferase activity ($t=0$ level). For each time point, 150 µl samples with the thus predetermined dilution of the crude lysate were prepared in triplicate and stored on ice. The samples were incubated at 45 °C for varying time periods, starting with the longest incubation time (30 min). At the end of the incubation time, all samples were placed on ice for 10 min. Next, A549 cells were exposed for 4 h at 37 °C/10 % CO_2 to 100 µl vector preparation, after which A549 cells received fresh culture medium. Twenty-four hours later, luciferase activity was determined by using a Steady-Glo kit (Promega) and the instructions provided.

Western blot analyses and RACE-PCR. PER.C6 cells were transfected with 2 µg each DNA construct and cells were harvested and lysed (20 mM Tris/HCl, 150 mM NaCl, 1 % deoxycholate, 1 % Tween 20) 48 h post-transfection. Cleared protein lysates were loaded on a 4–12 % Bis/Tris NuPage gel (polyacrylamide pre-cast mini-gel system; Invitrogen) in MES buffer (Invitrogen) and blotted onto a nitrocellulose membrane, after which the blot was incubated for 1 h with a 1 : 10 000-diluted polyclonal antibody raised in rabbits against culture filtrate protein of *M. tuberculosis*, kindly provided by John Belisle (Colorado State University, Fort Collins, CO, USA). After three wash steps, the presence of *M. tuberculosis* proteins on the membrane was visualized by using 1 : 10 000-diluted goat anti-rabbit IgG–horseradish peroxidase (Bio-Rad). The membrane was developed by using the enhanced chemiluminescence (ECLplus; Amersham Biosciences) system and instructions provided by the manufacturer.

To determine the transcription start sites of the pIX mRNA, the 5′ ends of pIX cDNA prepared from cultures infected with wild-type adenoviruses were amplified and sequenced. Infections with wild-type viruses were done on PER.C6/55K cells (m.o.i., 50 VP per cell). RNA was isolated 16 h post-infection by using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. At the end of the procedure, RNA was dissolved in 100 % formamide. A GeneRacer kit (Invitrogen) was used to amplify the 5′ end of full-length pIX transcripts. RNA (5 µg) was purified from formamide by sodium acetate precipitation as described in the GeneRacer protocol. cDNA was synthesized by reverse transcription using SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer's protocol and a gene-specific reverse primer for pIX that, for adenovirus types 3, 7 and 35, was called #465 (3′-CATTCTGACGAACTCCTGCC-5′), for type 11 #342 (3′-GGCGGGTTGAACGGGTCTTCCA-5′), for type 4 #497 (3′-TGCAGCTGAGCCACCTGCTG-5′), for type 5 #351 (3′-AGGGGAGGAAGCCTTCAGG-5′), for type 12 #468 (3′-GCAGGKGCSACAGGWCGACC-5′) and for type 26 #467 (3′-CATTCTGACGHACTCCYGCC-5′). With the exception of Ad5 and Ad11, 1 µl cDNA was used as a template for PCR to amplify the pIX fragment. This PCR was done by using *Pwo* DNA polymerase (Roche) with the GeneRacer 5′ primer from the kit (specific for the oligo ligated to the 5′ end of the mRNA) and the gene-specific reverse primers. Reaction started with denaturation at 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 2 min, and finished by elongation at 68 °C for 8 min. The resulting DNA fragments were size-separated by electrophoresis on a 1·0 % agarose gel. Specific fragments were excised and purified from the agarose gel. The purified DNA fragments were cloned into the pCR4Blunt-TOPO vector (Invitrogen) according to the manufacturer's protocol and sequenced.

Mouse immunizations and T-cell assays. C57BL/6 mice, 6–8 weeks old, were obtained from Harlan (Zeist) and were vaccinated intramuscularly with rAd35.TB-S vaccine ($n=5$ per group) using an increasing dose range (10^7–10^{10} VP). Two weeks post-immunization, a single-cell suspension was prepared by grinding spleens through a cell strainer (Falcon). After centrifugation (5 min and 515 ***g*** at 4 °C), the cell pellet was resuspended in ACL lysis buffer (BioWhittaker) and incubated for 2 min at room temperature. After washing, cell suspension was filtered through a pre-separation filter (MACS) and cell concentration was adjusted to 10^7 cells ml^{-1}. Mice receiving PBS served as a negative control in all immunization experiments and read-outs. Antigen-specific cellular immune responses were determined by using intracellular gamma interferon (IFN-γ) staining (ICS). Hereto, 10^6 splenocytes of each mouse were plated per well in 96-well plates and were subsequently stimulated in duplicate with overlapping peptide pool (final concentration, 2 µg each peptide ml^{-1}) in the presence of 1 : 1000-diluted anti-mouse–CD49d and anti-mouse–CD28 co-stimulatory antibodies (Pharmingen). The peptide pools consisted of 15-mer peptides spanning entire antigen-coding domains, with 10-mer (Ag85B, 55 peptides) or 11-mer (Ag85A, 71 peptides and TB10.4, 21 peptides) overlapping sequences. As a positive control, samples were stimulated with 50 ng phorbol-12-myristate-13-acetate ml^{-1}, 2 µg ionomycin ml^{-1} (final concentration), whilst incubation with medium alone served as negative control. After 1 h stimulation at 37 °C, 1 : 200-diluted GolgiPlug (Pharmingen) was added to block secretion and incubation was continued for an additional 5 h. Corresponding duplicate samples were pooled and processed for FACS analysis essentially as described previously (Ophorst *et al.*, 2006). At least 10 000 $CD8^+$ or $CD4^+$ cells were recorded for each individual sample. Biostatistical analyses were performed by using Student's *t*-test.

Tetrameric H-$2D^b$ complexes folded around the immunodominant SIVGag AL11 epitope (AAVKNWMTQTL) (Barouch *et al.*, 2004) were prepared and utilized to stain peptide-specific $CD8^+$ T lymphocytes as described previously (Barouch *et al.*, 2003). Samples were analysed by two-colour flow cytometry on a FACScalibur (Becton Dickinson) using CellQuestPro software. Gated $CD8^+$ T lymphocytes were examined for staining with the D^b–AL11 tetramer.

RESULTS

pIX gene regulation in human adenoviruses

We have previously reported the generation of E1-deleted rAd35 vectors by using PER.C6/55K cells, i.e. PER.C6 cells engineered to express the E1B-55K protein derived from Ad35. We consistently observed that, depending on the length of the inserted transgene and thus the total genome size of rAd35 (with or without the E3 region), severe deletions within the transgene region occurred in rAd11 vectors (data not shown) and rAd35 vectors (Fig. 1a), as witnessed by smaller DNA fragments detected by using insert-specific

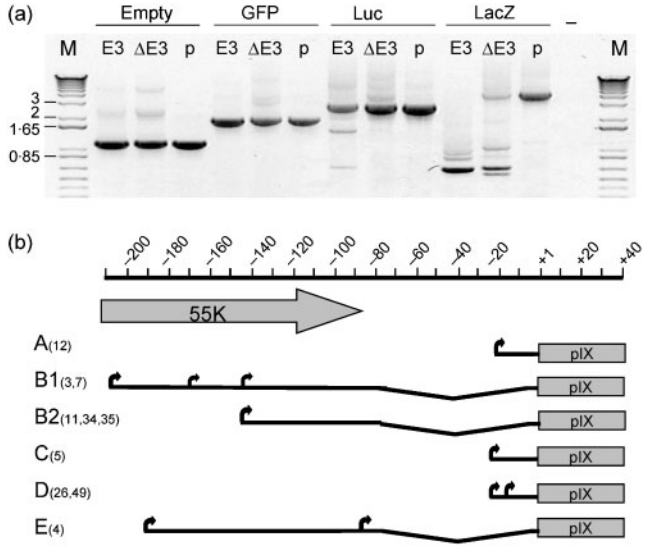

Fig. 1. Relationship between insert stability and Ad35 genome size. (a) Analysis of the transgene region at passage 2 in E1-deleted rAd35 viruses carrying no transgene (empty), eGFP, luciferase or *lacZ*. Viruses either contain the complete E3 region (E3) or a 2·6 kb deletion in E3 (ΔE3). The respective pAdapt35 plasmid was used as control (p). Transgene-deleted rAd35 inserts are recognized as DNA fragments smaller than the plasmid control. (b) Schematic representation showing 5′-RACE results identifying pIX transcription start sites (indicated as arrows) in members of human adenoviruses derived from different subgroups (A–E).

PCR. Sequence analyses of several insert-deletion-mutant rAd35 vectors demonstrated that, in all of these viable mutants, the CMV promoter was placed directly in front of the pIX gene. The pIX gene encodes a protein that, in the context of Ad5, is important for packaging of full-length adenovirus genomes (Bett *et al.*, 1993) and thus we hypothesized that pIX protein expression might be limited in E1-deleted rAd35 vectors. Sequence analyses indicated four putative pIX promoters in B2-group viruses, which all proved to be located within the E1B region of the Ad35 virus. To map the 5′ transcription start sites within human adenoviruses, we performed 5′-RACE analyses and identified that members of human subgroups A, C and D all contain the pIX promoter just upstream of the pIX coding domain and downstream of the E1B coding domain. In contrast, subgroup B and E adenoviruses utilize pIX promoter elements located within the E1B region (Fig. 1b). In the case of B2-group adenovirus types 11, 34 and 35, one of four putative promoters coincided with the sole pIX cap site within Ad35 virus (probability score 0·63, sequence ranging from nt 3234 to 3488: CCGGTGTGTGTAGATGTGACCGAAGATCTCAGACCGGATCATTTGGTTAT). The four putative promoters were identified by using a computational-prediction program for gene structure and regulation (http://www.fruitfly.org/seq_tools/promoter.html).

pIX gene expression, rAd35 vector stability and packaging size

Next, we investigated the relationship between rAd35 capsid stability and pIX protein expression. Hereto, we employed an assay based on the observation that, at elevated temperatures (45–48 °C), Ad5 adenoviral capsids lacking pIX protein are destroyed, but adenoviral capsids containing pIX protein are not (Colby & Shenk, 1981). As shown in Fig. 2(a), incubation for prolonged periods at 45 °C had no effect on an rAd35 vector carrying the pIX coding region and the pIX promoter element (Ad35.E1B). In contrast, the capsid structure of rAd35 vectors deleted for the pIX promoter (Ad35.ΔE1) was impaired severely, as witnessed by a 50 % reduction of luciferase-gene activity after incubating the vector for <10 min at 45 °C. An rAd35 vector carrying the E1B region, but deleted for the pIX coding region (Ad35.E1B.ΔpIX), proved severely temperature-sensitive, thus linking the capsid instability directly to low-level or lacking pIX expression. Subsequent, detailed pIX promoter-mapping studies (data not shown) identified that thermostable rAd35 vectors could be generated when retaining, in E1-deleted rAd35 vectors, a 243 bp fragment upstream of the pIX start codon, marked at the 5′ end by a *Bsu*36I restriction site (Ad35Bsu). This 243 bp fragment includes 167 bp from the 3′ end of the Ad35-E1B sequence, as well as a 76 bp non-coding Ad35 genome sequence (Fig. 2b). A head-to-head comparison between rAd35 vectors either carrying the 167 bp E1B fragment or not showed that the presence of the minimal pIX promoter in the novel rAd35 vectors did not influence CMV promoter activity, as indicated by the level of luciferase-transgene activity (Fig. 2c). Insert-integrity analyses using CsCl gradient-purified rAd35 vectors demonstrated that the novel, stabilized rAd35 vectors can accommodate at least 5 kb foreign DNA successfully, as no deletion fragments were detected after five rounds of vector propagation by using the highly sensitive insert-integrity PCR assay (Fig. 2d).

Manufacture of rAd35 on Ad5 E1-complementing PER.C6 packaging cells

The pAdapt35 plasmid modified to contain the minimal pIX promoter was used to generate two different rAd35 vectors carrying green fluorescent protein (GFP). One vector contained the wild-type organization of the Ad35 E4 region and therefore needed to be produced on the PER.C6/55K cell line (Vogels *et al.*, 2003). Within the second vector, the Ad35 E4 sequences encoding a protein called Orf6 were replaced by Ad5 E4-Orf6 sequences, as within the normal adenoviral life cycle, proteins E1B-55K and E4-Orf6 form a complex that is pivotal for high-level late-gene expression by influencing the preferential transport of viral mRNAs over cellular mRNAs from the nucleus to the cytoplasm (Rubenwolf *et al.*, 1997; Weigel & Dobbelstein, 2000). We thus hypothesized that functional interaction of this Ad5Orf6 protein with Ad5-derived E1 proteins present in PER.C6 cells might result in efficient manufacture of E1-deleted rAd35 vectors on PER.C6 cells. As shown in

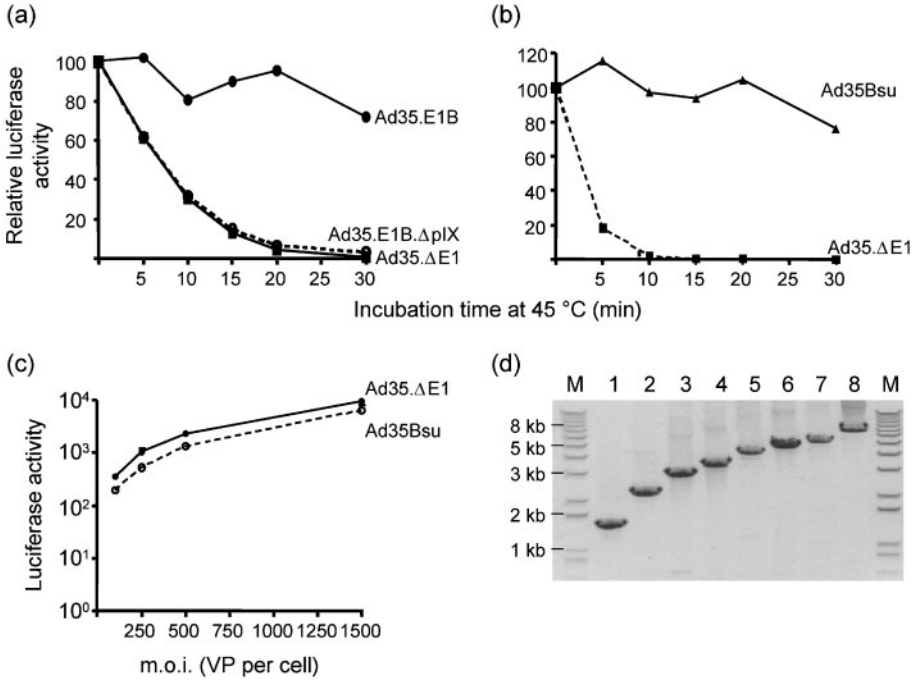

Fig. 2. Correlation between pIX protein level and rAd35 capsid stability. (a) Capacity of Ad35.ΔE1, Ad35.E1B and rAd35.E1B.ΔpIX vectors to transfer the luciferase marker gene into A549 cells after incubating vectors at 45 °C for varying time points up to 30 min. Luciferase-activity measurements, expressed relative to the luciferase activity obtained at time point 0, were measured in human-derived A549 cells 48 h after virus exposure. (b) Experiment identical to that described in (a), but comparing rAd35 vectors fully deleted for E1 (Ad35.ΔE1) with an rAd35 vector containing the minimal pIX promoter (Ad35Bsu). (c) Comparison of dose-dependent luciferase-gene expression obtained in human A549 cells from rAd35.ΔE1 and rAd35.Bsu vectors. (d) Transgene PCR (including 1·4 kb CMV promoter and part of SV40 pA) analyses of rAd35.Bsu vectors carrying transgenes of 0 kb (lane 1), 0·5 kb (lane 2), 1·6 kb (lane 3), 2 kb (lane 4), 2·7 kb (lane 5), 3 kb (lane 6), 3·6 (lane 7) and 5·1 kb (lane 8). Vector analyses were performed after five propagation rounds by using CsCl-purified vector batches.

Fig. 3(a), inoculation of human A549 cells with supernatant derived from rAd35-35Orf6-infected PER.C6/55K cells resulted in GFP-positive A549 cells, indicative of the presence of viable virus. As expected, supernatant derived from rAd35-35Orf6-infected PER.C6 cells did not yield GFP-positive A549 cells. In contrast, inoculation of human A549 cells with supernatant derived from rAd35-5Orf6-infected PER.C6/55K and PER.C6 cells yielded GFP-positive A549 cells. Next, a head-to-head comparison was performed to identify whether vector yield or vector integrity was compromised by using rAd35-35Orf6 as opposed to rAd35-5Orf6. As shown in Fig. 3(b), vector yield, expressed as VP (ml cell lysate)$^{-1}$, was significantly (two-tailed *t*-test, $P<0{\cdot}0001$) higher using the rAd35-5Orf6 vector on PER.C6 compared with rAd35-35Orf6 yield on PER.C6/55K cells. Also, batch quality was improved, as the VP : p.f.u. ratio was consistently lower for rAd35-5Orf6 produced on PER.C6 (mean $\pm$ SD, $9{\cdot}7\pm1{\cdot}8$ VP : p.f.u.; $n=9$) compared with rAd35-35Orf6 produced on PER.C6/55K (mean $\pm$ SD, $19{\cdot}4\pm8{\cdot}9$ VP : p.f.u.; $n=11$). This improved VP : p.f.u. ratio resulted in significantly increased (two-tailed *t*-test, $P<0{\cdot}0001$) GFP-gene transfer and expression after inoculation of human A549 cells with equal amounts of VP per cell of rAd35-5Orf6 vector or rAd35-35Orf6 inoculation (Fig. 3c). Finally, we investigated whether replacing the 35Orf6 with the 5Orf6 region influenced gene expression and, consequently, insert-specific immune responses. Hereto, groups of naïve C57BL/6 mice ($n=8$ per group) were vaccinated with 10^9 VP rAd35-35Orf6 and rAd35-5Orf6 vector carrying SIVgag antigen. Gag-specific cellular immune responses were assessed by D^b–AL11 tetramer-binding assays following immunization. As shown in Fig. 3(d), no statistically significant differences (ANOVA) were observed between the two rAd35-based vaccines and thus it could be concluded that the presence of the Ad5-derived Orf6 protein does not compromise either level or longevity of antigen-specific immune responses. Together, the novel genetic modifications within the Ad35 genome, i.e. retaining the pIX promoter element and replacing the 35Orf6 sequence with the 5Orf6 sequence, are pivotal to ensure stable insertion of large DNA fragments and excellent yield of replication-incompetent rAd35 vaccines. Currently, with PER.C6 cells growing at 10 million cells ml^{-1} at the 10 l scale, 10^5 VP rAd35 per cell and purification recovery at

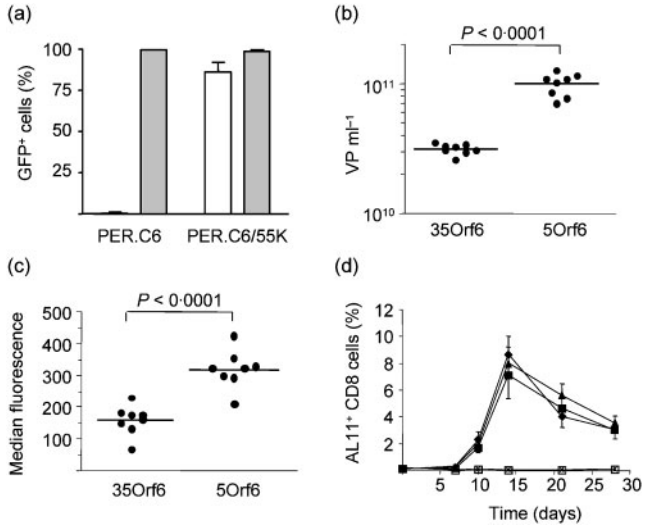

Fig. 3. Replication of ΔE1-rAd35 vectors on PER.C6 or PER.C6/55K cells. (a) Percentage of eGFP-positive A549 cells at 48 h after exposure of cells to 50 μl crude lysate. The crude lysate was prepared from E1-deleted Ad35 vectors (empty bars) or from Ad35-5Orf6 vectors (shaded bars) propagated for 3 days on PER.C6 or PER.C6/55K cells. GFP expression was assessed by using FACS (non-transduced cells were used to set the background value at 1 %). The percentage of GFP^+ cells obtained 48 h after virus exposure ($n=8$) is shown. (b) Ad35 (35Orf6) and Ad35-5Orf6 (5Orf6) vectors carrying GFP were produced on PER.C6/55K and PER.C6, respectively ($n=8$), and yield was determined after pre-purification by HPLC. (c) Ad35 (35Orf6) vectors and Ad35-5Orf6 (5Orf6) vectors carrying GFP were used to infect A549 cells with 500 VP per cell ($n=8$). Median fluorescence was determined after 24 h by using FACS. (d) Immunogenicity of rAd35 (◆), rAd35.ΔE4.35Orf6/7 (▲) and rAd35.ΔE4.5Orf6/7 (■) vectors expressing SIVgag or a sham (☒) vector control in naïve C57BL/6 mice (dose, 10^9 VP; intramuscular immunization). SIVgag-specific $CD8^+$ T-lymphocyte responses were assessed by D^b–AL11 tetramer-binding assays at multiple time points following vaccination.

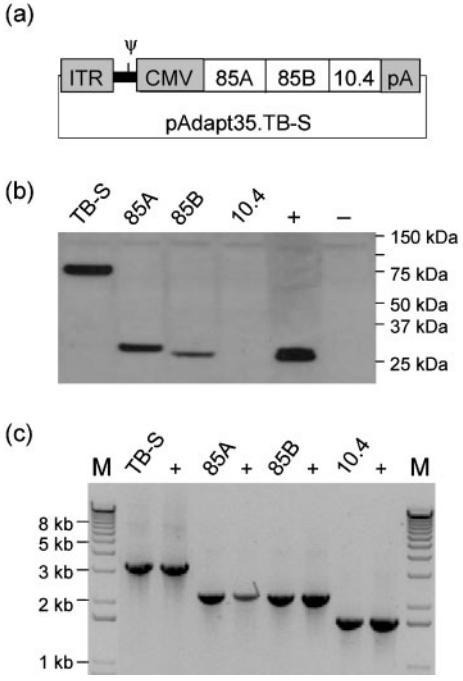

Fig. 4. Stable multi-gene rAd35 vaccine. (a) Schematic representation of the pAdapt35.Bsu plasmid used to generate the rAd35.TB-S vaccine. The positions of the left inverted terminal repeat (ITR) and packaging signal (Ψ) are shown. Transcription of the multi-gene is under the control of the CMV promoter and SV40 polyadenylation (pA) sequence. (b) Plasmids containing single *M. tuberculosis* antigens or TB-S were transfected into PER.C6 cells and expression was analysed 2 days later by Western blot analysis. As positive control (+), 20 μg Ag85 complex purified protein was used, whereas untransfected PER.C6 lysate served as negative (–) control. (c) PCR analyses of the transgene region of purified rAd35 vaccine batches expressing TB-S or single *M. tuberculosis* antigen constructs. As positive controls (+), pAdapt35 DNA constructs were used as template for PCR.

50 % (data not shown), it is envisioned that millions of rAd35 vector-based vaccine dosages can be manufactured.

Design of a multi-antigen rAd35 vaccine

To evaluate insert capacity of the improved rAd35 vector, a multi-antigen was designed expressing a fusion protein of three major *M. tuberculosis* antigens (Fig. 4a). Transient transfection of the DNA construct into PER.C6 cells and Western blot analyses demonstrated proper expression of the polyprotein, although the polyclonal antibody preparation did not detect TB10.4 (Fig. 4b). The TB-S construct was subsequently used to generate the rAd35.TB-S vaccine. As shown in Fig. 4(c), insert-integrity PCR analyses demonstrated that TB-S could be generated stably within the context of the rAd35 vector, resulting in $>10^{13}$ VP after laboratory-scale production and CsCl purification.

Immunogenicity of the multi-antigen rAd35 vaccine

To determine the induction of T-cell responses against *M. tuberculosis* antigens, as well as the immunological antigen recognition using a triple-antigen construct, mice were immunized with 10^7–10^{10} VP rAd35.TB-S vaccine. As shown in Fig. 5(a, b), significant $CD4^+$ T-cell responses were induced against Ag85A (two-tailed *t*-test, $P<0{\cdot}002$) and Ag85B (two-tailed *t*-test, $P<0{\cdot}001$) compared with the control group, 2 weeks after immunization using rAd35 vaccine dosages exceeding 10^7 VP. $CD4^+$ T-cell responses could not be detected against TB10.4, even at a vaccine dosage of 10^{10} VP (Fig. 5c). Also, strong $CD8^+$ T-cell responses against Ag85A, Ag85B and TB10.4 (Fig. 5d–f, respectively) were obtained at vaccine dosages exceeding 10^8 VP, which were significantly higher (two-tailed *t*-test, $P<0{\cdot}0001$) compared with the control group. For all

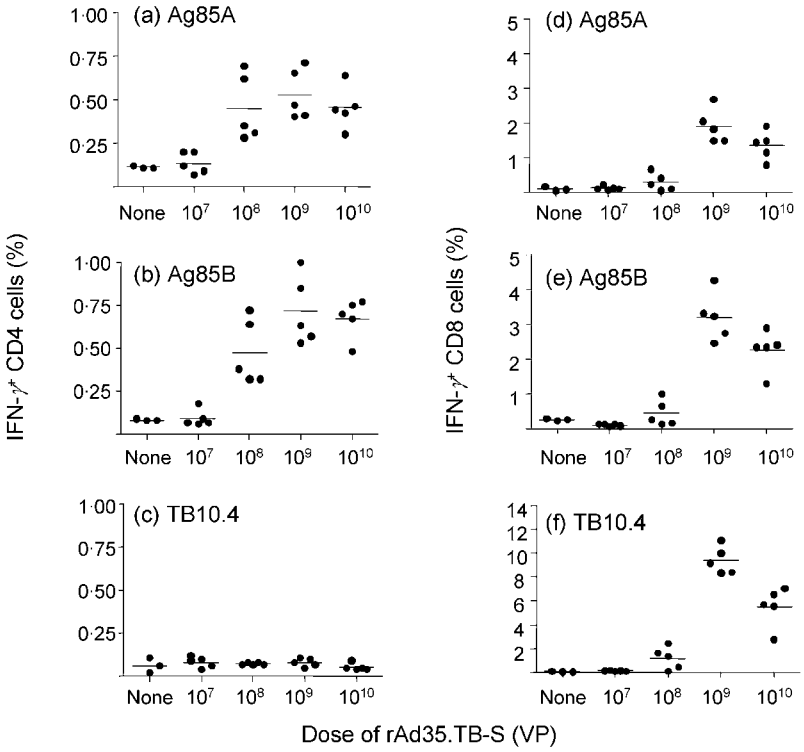

Fig. 5. Induction of T-cell response by using rAd35.TBS vaccine. C57BL/6 mice were vaccinated intramuscularly with increasing doses of Ad35.TB-S. Splenocytes, isolated 2 weeks after immunization, were stimulated *in vitro* with overlapping peptide pools corresponding to Ag85A (a, d), Ag85B (b, e) or TB 10.4 (c, f) proteins. IFN-γ production of T cells was determined by intracellular FACS staining.

antigens, the optimal vaccine dose for $CD8^+$ T-cell responses was achieved at 10^9 VP.

DISCUSSION

The studies described aimed to solve the observed genome-instability and scalability issues surrounding the use of the rAd35 vaccine carrier.

Evidence that impaired regulation of pIX expression caused the limited packaging capacity included lack of an SP1-binding site and TATA box in the intergenic region between the E1B-55K and pIX coding regions in Ad35 and Ad11, in contrast to Ad5 (Babiss & Vales, 1991), and observations that Ad35 vectors carrying the complete E1B region did not show deletions of the transgene region, even at a total genome size of 105 % of wild-type genome length. Lack of available tools to quantify pIX expression levels in the Ad35 capsid forced us to investigate the role of pIX in capsid stability by thermostability analyses. For Ad5 viruses, it has been reported that lack of the pIX protein resulted in viruses that are heat-labile (Colby & Shenk, 1981) and have a limited packaging capacity (Ghosh-Choudhury *et al.*, 1987), a characteristic that can be partially overcome by over-expressing pIX proteins in complementing cell lines (Caravokyri & Leppard, 1995). The pIX protein has been suggested to have additional functions in type 5 transcription activation and nuclear remodelling (Lutz *et al.*, 1997; Rosa-Calatrava *et al.*, 2001, 2003), although the significance of these functions for replication in cell lines is currently not clear (Sargent *et al.*, 2004). Our results show that, like subgroup C virus, the presence of pIX protein is required to obtain capsid-stable B2-group viruses. Based on the data obtained, it cannot be concluded whether the spontaneous formation of Ad35 deletion mutants, relocating the CMV promoter just upstream of the pIX coding region, occurs via a mechanism simply selecting deleted viral genomes that form spontaneously during normal virus replication, or whether lack of pIX expression forces the formation of such deletion mutants. Thus, a definite role of the pIX protein in adenovirus genome-replication efficiency remains to be investigated.

We scanned sequences of human (types 4, 5, 12, 35, 40 and 49), simian (types A, 1, 3, 18, 21–25, 39 and Pan5–7), canine (types 1 and 2), bovine (types 2 and 3) and porcine (type 5) adenoviruses for the presence of pIX promoter sequences. Serotypes that did not contain the promoter in the intergenic region included Pan5–7, simian 21–25 and human types 4 and 35. All other serotypes included in this analysis did contain the promoter sequences in this genomic region. The emergence of two groups based on promoter sequences within the analysed region coincides nicely with clustering of the adenoviral regions in a phylogenetic tree (data not shown). Also, the human adenoviruses analysed for the presence or absence of a pIX transcription start site in the intergenic 55K-pIX region confirmed the above phylogenetic analyses. Together, the sequence analyses suggest that other adenoviruses, including several simian viruses that are currently being developed as vector systems (Cohen *et al.*, 2002; Xiang *et al.*, 2002; Pinto *et al.*, 2004), contain a pIX promoter in the E1B-55K region and, as such, may

experience genome instability in E1-deleted formats. Other animal adenoviruses, including types derived from bovine, porcine and canine adenoviruses, fall in the same group as Ad5, i.e. had a high probability of the presence of a pIX transcription start site in the intergenic region.

We next sought to achieve production of E1-deleted rAd35 vector on PER.C6 cells rather than to use the novel, specifically engineered PER.C6/55K cell line described previously (Vogels *et al.*, 2003). Reasons for this included the excellent documentation and safety data on the PER.C6 cell line, the scalability of the cells without the need for either microcarrier support or serum components and the know-how on growing PER.C6 cells in batch, fed-batch and continuous-perfusion systems (Jones *et al.*, 2003). Based on our previous work demonstrating that PER.C6 cells modified to express Ad35 E1B-55K proteins could complement E1-deleted Ad35 and Ad11 vectors, we postulated that a poor interaction between Ad5-derived E1B-55K proteins with Ad35-derived E4-Orf6 proteins causes the lack of replication of E1-deleted Ad35 and Ad11 vectors on PER.C6 (Vogels *et al.*, 2003). Within the normal adenoviral life cycle, proteins E1B-55K and E4-Orf6 form a complex that is pivotal for high-level late-gene expression by influencing the preferential transport of viral mRNAs over cellular mRNAs from the nucleus to the cytoplasm (Rubenwolf *et al.*, 1997; Weigel & Dobbelstein, 2000). The E1B-55K proteins, however, also have other functions, including downregulation of p53 proteins to prevent E1A-induced apoptosis (Weigel & Dobbelstein, 2000). This, and possibly other unknown functions, may limit the maximum level of constitutive expression of E1B-55K proteins in mammalian cells in general and E1-immortalized cells in particular. The latter could lead to a suboptimal level of E1B-55K expression that, coupled with a poor interaction with E4-Orf6 protein derived from the B2 group, could explain the observed variations in vaccine-batch quality using PER.C6/55K. The method of swapping native adenovirus Orf6 sequences for Ad5 E4-Orf6 in the backbone B2-group vectors, facilitating enhanced replication and improving quality (improved VP : p.f.u. ratio) on Ad5 E1-expressing complementing cell lines, is likely to be a universal method, as replication-deficient subgroup D viruses carrying Ad5 E4-Orf6 sequence also demonstrate enhanced replication on PER.C6 cells (data not shown).

To investigate whether the novel rAd35 vector-genome modifications impact on potency of immune responses, rAd35.TB-S was generated. A multi-antigen approach was chosen, as such a vaccine is expected to elicit broader immune responses than single-antigen vaccines, thus being potentially more effective in preventing immune escape of a pathogen. Whilst in theory, advantages of multi-antigen-based vaccines may be clear, fusion of antigens could compromise protein expression or intracellular protein trafficking, in turn compromising either level or breadth of immune responses. Here, we present data showing that both DNA and adenoviral vectors can be generated stably and express three antigens derived from *M. tuberculosis*, resulting in $CD4^+$ and $CD8^+$ T-cell responses against multiple antigens.

In conclusion, the improvements on the backbone described here pave the way towards industrial-scale manufacturing of rAd35 vectors carrying large and complex foreign DNA inserts by ensuring genome stability and production on existing Ad5 E1-complementing cell lines, such as PER.C6. These parameters, further coupled to the low seroprevalence in human populations worldwide and strong induction of antigen-specific cellular immunity, qualify rAd35 as a highly promising vaccine carrier for further vaccine development.

ACKNOWLEDGEMENTS

The authors thank Rob Hoeben (LUMC, The Netherlands) for construction of the Ad35.ΔpIX plasmid and June Wang (Aeras) for stimulating discussions on TB antigen and vector design. The authors also thank Gerrit Jan Weverling (Crucell Holland BV) for statistical analyses of the data.

REFERENCES

Arribillaga, L., de Cerio, A. L. D., Sarobe, P. & 9 other authors (2002). Vaccination with an adenoviral vector encoding hepatitis C virus (HCV) NS3 protein protects against infection with HCV-recombinant vaccinia virus. *Vaccine* **21**, 202–210.

Babiss, L. E. & Vales, L. D. (1991). Promoter of the adenovirus polypeptide IX gene: similarity to E1B and inactivation by substitution of the simian virus 40 TATA element. *J Virol* **65**, 598–605.

Barouch, D. H. & Nabel, G. J. (2005). Adenovirus vector-based vaccines for human immunodeficiency virus type 1. *Hum Gene Ther* **16**, 149–156.

Barouch, D. H., McKay, P. F., Sumida, S. M. & 8 other authors (2003). Plasmid chemokines and colony-stimulating factors enhance the immunogenicity of DNA priming-viral vector boosting human immunodeficiency virus type 1 vaccines. *J Virol* **77**, 8729–8735.

Barouch, D. H., Pau, M. G., Custers, J. H. H. V. & 15 other authors (2004). Immunogenicity of recombinant adenovirus serotype 35 vaccine in the presence of pre-existing anti-Ad5 immunity. *J Immunol* **172**, 6290–6297.

Bett, A. J., Prevec, L. & Graham, F. L. (1993). Packaging capacity and stability of human adenovirus type 5 vectors. *J Virol* **67**, 5911–5921.

Caravokyri, C. & Leppard, K. N. (1995). Constitutive episomal expression of polypeptide IX (pIX) in a 293-based cell line complements the deficiency of pIX mutant adenovirus type 5. *J Virol* **69**, 6627–6633.

Casimiro, D. R., Chen, L., Fu, T.-M. & 36 other authors (2003). Comparative immunogenicity in rhesus monkeys of DNA plasmid, recombinant vaccinia virus, and replication-defective adenovirus vectors expressing a human immunodeficiency virus type 1 *gag* gene. *J Virol* **77**, 6305–6313.

Cohen, C. J., Xiang, Z. Q., Gao, G.-P., Ertl, H. C. J., Wilson, J. M. & Bergelson, J. M. (2002). Chimpanzee adenovirus CV-68 adapted as a gene delivery vector interacts with the coxsackievirus and adenovirus receptor. *J Gen Virol* **83**, 151–155.

Colby, W. W. & Shenk, T. (1981). Adenovirus type 5 virions can be assembled in vivo in the absence of detectable polypeptide IX. *J Virol* **39**, 977–980.

Fallaux, F. J., Kranenburg, O., Cramer, S. J., Houweling, A., Van Ormondt, H., Hoeben, R. C. & Van Der Eb, A. J. (1996). Characterization of 911: a new helper cell line for the titration and propagation of early region 1-deleted adenoviral vectors. *Hum Gene Ther* **7**, 215–222.

Fallaux, F. J., Bout, A., van der Velde, I. & 9 other authors (1998). New helper cells and matched early region 1-deleted adenovirus vectors prevent generation of replication-competent adenoviruses. *Hum Gene Ther* **9**, 1909–1917.

Ghosh-Choudhury, G., Haj-Ahmad, Y. & Graham, F. L. (1987). Protein IX, a minor component of the human adenovirus capsid, is essential for the packaging of full length genomes. *EMBO J* **6**, 1733–1739.

Holterman, L., Vogels, R., van der Vlugt, R. & 15 other authors (2004). Novel replication-incompetent vector derived from adenovirus type 11 (Ad11) for vaccination and gene therapy: low seroprevalence and non-cross-reactivity with Ad5. *J Virol* **78**, 13207–13215.

Jaiswal, S., Khanna, N. & Swaminathan, S. (2003). Replication-defective adenoviral vaccine vector for the induction of immune responses to dengue virus type 2. *J Virol* **77**, 12907–12913.

Jones, D., Kroos, N., Anema, R. & 12 other authors (2003). High-level expression of recombinant IgG in the human cell line PER.C6. *Biotechnol Prog* **19**, 163–168.

Kostense, S., Koudstaal, W., Sprangers, M. & 8 other authors (2004). Adenovirus types 5 and 35 seroprevalence in AIDS risk groups supports type 35 as a vaccine vector. *AIDS* **18**, 1213–1216.

Lemckert, A. A. C., Sumida, S. M., Holterman, L. & 10 other authors (2005). Immunogenicity of heterologous prime-boost regimens involving recombinant adenovirus serotype 11 (Ad11) and Ad35 vaccine vectors in the presence of anti-Ad5 immunity. *J Virol* **79**, 9694–9701.

Liu, R.-Y., Wu, L.-Z., Huang, B.-J. & 9 other authors (2005). Adenoviral expression of a truncated S1 subunit of SARS-CoV spike protein results in specific humoral immune responses against SARS-CoV in rats. *Virus Res* **112**, 24–31.

Lutz, P., Rosa-Calatrava, M. & Kedinger, C. (1997). The product of the adenovirus intermediate gene IX is a transcriptional activator. *J Virol* **71**, 5102–5109.

Maizel, J. V., Jr, White, D. O. & Scharff, M. D. (1968). The polypeptides of adenovirus. I. Evidence for multiple protein components in the virion and a comparison of types 2, 7A, and 12. *Virology* **36**, 115–125.

Ophorst, O. J. A. E., Radošević, K., Havenga, M. J. E., Pau, M. G., Holterman, L., Berkhout, B., Goudsmit, J. & Tsuji, M. (2006). Immunogenicity and protection of a recombinant human adenovirus serotype 35-based malaria vaccine against *Plasmodium yoelii* in mice. *Infect Immun* **74**, 313–320.

Phillpotts, R. J., O'Brien, L., Appleton, R. E., Carr, S. & Bennett, A. (2005). Intranasal immunisation with defective adenovirus serotype 5 expressing the Venezuelan equine encephalitis virus E2 glycoprotein protects against airborne challenge with virulent virus. *Vaccine* **23**, 1615–1623.

Pinto, A. R., Fitzgerald, J. C., Giles-Davis, W., Gao, G. P., Wilson, J. M. & Ertl, H. C. J. (2003). Induction of $CD8^+$ T cells to an HIV-1 antigen through a prime boost regimen with heterologous E1-deleted adenoviral vaccine carriers. *J Immunol* **171**, 6774–6779.

Pinto, A. R., Fitzgerald, J. C., Gao, G. P., Wilson, J. M. & Ertl, H. C. J. (2004). Induction of $CD8^+$ T cells to an HIV-1 antigen upon oral immunization of mice with a simian E1-deleted adenoviral vector. *Vaccine* **22**, 697–703.

Rosa-Calatrava, M., Grave, L., Puvion-Dutilleul, F., Chatton, B. & Kedinger, C. (2001). Functional analysis of adenovirus protein IX identifies domains involved in capsid stability, transcriptional activity, and nuclear reorganization. *J Virol* **75**, 7131–7141.

Rosa-Calatrava, M., Puvion-Dutilleul, F., Lutz, P., Dreyer, D., de Thé, H., Chatton, B. & Kedinger, C. (2003). Adenovirus protein IX sequesters host-cell promyelocytic leukaemia protein and contributes to efficient viral proliferation. *EMBO Rep* **4**, 969–975.

Rubenwolf, S., Schütt, H., Nevels, M., Wolf, H. & Dobner, T. (1997). Structural analysis of the adenovirus type 5 E1B 55-kilodalton–E4orf6 protein complex. *J Virol* **71**, 1115–1123.

Sargent, K. L., Meulenbroek, R. A. & Parks, R. J. (2004). Activation of adenoviral gene expression by protein IX is not required for efficient virus replication. *J Virol* **78**, 5032–5037.

Seaman, M. S., Xu, L., Beaudry, K. & 11 other authors (2005). Multiclade human immunodeficiency virus type 1 envelope immunogens elicit broad cellular and humoral immunity in rhesus monkeys. *J Virol* **79**, 2956–2963.

Shabram, P. W., Giroux, D. D., Goudreau, A. M. & 7 other authors (1997). Analytical anion-exchange HPLC of recombinant type-5 adenoviral particles. *Hum Gene Ther* **8**, 453–465.

Shanley, J. D. & Wu, C. A. (2003). Mucosal immunization with a replication-deficient adenovirus vector expressing murine cytomegalovirus glycoprotein B induces mucosal and systemic immunity. *Vaccine* **21**, 2632–2642.

Shiver, J. W. & Emini, E. A. (2004). Recent advances in the development of HIV-1 vaccines using replication-incompetent adenovirus vectors. *Annu Rev Med* **55**, 355–372.

Shiver, J. W., Fu, T.-M., Chen, L. & 49 other authors (2002). Replication-incompetent adenoviral vaccine vector elicits effective anti-immunodeficiency-virus immunity. *Nature* **415**, 331–335.

Sullivan, N. J., Geisbert, T. W., Geisbert, J. B., Xu, L., Yang, Z.-Y., Roederer, M., Koup, R. A., Jarhling, P. B. & Nabel, G. J. (2003). Accelerated vaccination for Ebola virus haemorrhagic fever in non-human primates. *Nature* **424**, 681–684.

Sumida, S. M., Truitt, D. M., Lemckert, A. A. C. & 15 other authors (2005). Neutralizing antibodies to adenovirus serotype 5 vaccine vectors are directed primarily against the adenovirus hexon protein. *J Immunol* **174**, 7179–7185.

Tan, Y., Hackett, N. R., Boyer, J. L. & Crystal, R. G. (2003). Protective immunity evoked against anthrax lethal toxin after a single intramuscular administration of an adenovirus-based vaccine encoding humanized protective antigen. *Hum Gene Ther* **14**, 1673–1682.

Vogels, R., Zuijdgeest, D., van Rijnsoever, R. & 20 other authors (2003). Replication-deficient human adenovirus type 35 vectors for gene transfer and vaccination: efficient human cell infection and bypass of preexisting adenovirus immunity. *J Virol* **77**, 8263–8271.

Weigel, S. & Dobbelstein, M. (2000). The nuclear export signal within the E4orf6 protein of adenovirus type 5 supports virus replication and cytoplasmic accumulation of viral mRNA. *J Virol* **74**, 764–772.

Wu, L., Kong, W.-P. & Nabel, G. J. (2005). Enhanced breadth of CD4 T-cell immunity by DNA prime and adenovirus boost immunization to human immunodeficiency virus Env and Gag immunogens. *J Virol* **79**, 8024–8031.

Xiang, Z., Gao, G., Reyes-Sandoval, A., Cohen, C. J., Li, Y., Bergelson, J. M., Wilson, J. M. & Ertl, H. C. J. (2002). Novel, chimpanzee serotype 68-based adenoviral vaccine carrier for induction of antibodies to a transgene product. *J Virol* **76**, 2667–2675.