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. Sadoff2 and J. Goudsmit1

Correspondence
M. Havenga
m.havenga@crucell.com

1Crucell Holland BV, PO Box 2048, 2301 CA Leiden, The Netherlands
2AERAS Global TB Vaccine Foundation, 7500 Old Georgetown Road, Bethesda, MD 20814, USA
3Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, USA

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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.

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.,...
METHODS

Adenoviral-vector plasmids and TB-S construct. To generate a 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. AF271307). This plasmid, called pBr.Ad35.PRN, served as a template to amplify an Ad35 genome fragment of 18 kb (nt 30099–31880, containing a 3′ tail homologous to Ad5 sequences) by Ad5 sequence nt 32963–34077 (numbering as in GenBank accession no. M73260) and flanked by sequences homologous to the pIX coding region (nt 3484–3805 in Ad35), thereby removing most of the pIX coding region.

To create a new adaptor plasmid containing the pIX promoter, plasmid pAdapt35.PRI (Vogels et al., 2003) was modified. Hereto, an Ad5 restriction site located in the multiple cloning site behind the cytomegalovirus (CMV) promoter needed to be removed by partial digestion of Ad35 viruses or by TCID50 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 termination 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 assays. Viral DNA was isolated from crude lysate or purified vector batches by using a BglII, blunted with Klenow and further digested with AgeI. Ligation of the blunt Ad5 genome fragment to a Bsu36I–AgeI fragment corresponding to nt 3234–4251 in Ad5, in which the Bsu36I 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 SmaI and HindIII (nt 452–1338 in Ad35) were replaced by an AvrII–BglII 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 rAd35.E1B.PXI.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 41–1325) 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.

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Cell culture and vector generation, purification and titration. Cell lines PER.C6, PER.C6/55K and A549 were cultured at 37 °C/10% CO2 in Dulbecco’s modified Eagle’s medium (Life Technologies Inc.) containing 10% fetal bovine serum (Life Technologies Inc.) and further supplemented with 10 mM MgCl2 (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.PRI (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 cosm id containing Ad35 sequences (nt 3401–24649) called pWE.PIX.EcoRV; and (iii) plasmid pBr.Ad35.PRNAE3.50Ref. 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 TCID50 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).
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 (5′-GTAGGGTCAGCCTAGTTGTC-3′) and a reverse primer (#342) hybridizing to the pIX coding sequence (5′-GGCGGGTT-GAACGGGTTCCTTCA-3′).

PCR amplification was performed with Taq DNA polymerase (2.5 U; Invitrogen) in 1× supplied buffer, 0.2 mM dNTPs, 2 mM MgCl2, 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.

To determine viral thermostability, A549 cells were seeded at 5 × 104 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% CO2 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. Infection 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 was dissolved in 100% formamide. A GeneRacer kit (Invitrogen) was developed by using the enhanced chemiluminescence (ECLplus; Amersham Biosciences) system and instructions provided by the manufacturer.

**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 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 pCRBlunt-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 (107–1010 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 106 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, 105 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-2Dd complexes folded around the immunodominant SIgVag AL11 epitope (AAVKNWMQTQL) (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 D6–AL11 tetramer.
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: CCGGTGTGTTAGACTGTGACGGAAGATCCTAGACCGGATTTGGTAT). 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).

**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).

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 Bsi36I restriction site (Ad35.Bsu). 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
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, \(P<0.0001\)) 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 ± SD, 9.7 ± 1.8 VP : p.f.u.; \(n=9\)) compared with rAd35-35Orf6 produced on PER.C6/55K (mean ± SD, 19.4 ± 8.9 VP : p.f.u.; \(n=11\)). This improved VP: p.f.u. ratio resulted in significantly increased (two-tailed t-test, \(P<0.0001\)) 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\(^{9}\)-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
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<0.002) and Ag85B (two-tailed t-test, P<0.001) compared with the control group, 2 weeks after immunization using rAd35 vector 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<0.0001) compared with the control group. For all
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 55K-pIX 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 the 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.

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