CHAPTER 5

Generation of a novel replication-incompetent adenoviral vector derived from human adenovirus type 49: manufacture on PER.C6 cells, tropism and immunogenicity

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Abstract
Recombinant adenoviral vectors based on type 5 (rAd5) show great promise as vaccine carrier. However, neutralizing activity against Ad5 is prevalent and high-titred among human populations, and significantly dampens Ad5-based vaccine modalities. The generation of alternative adenoviral vectors with low seroprevalence thus receives much research attention. Here, it is shown that a member from human adenovirus subgroup D, i.e. Ad49, does not cross-react with Ad5 neutralizing activity, making it a candidate serotype for vector development. Therefore, a plasmid system that allows formation of replication-incompetent adenovirus serotype 49 vaccine vectors (rAd49) was constructed and it was demonstrated that rAd49 can be successfully propagated to high titres on existing Ad5.E1-complementing cell lines such as PER.C6. Using an rAd49 vector carrying the luciferase marker gene, detailed seroprevalence studies were performed, demonstrating that rAd49 has low seroprevalence and neutralizing antibody titres worldwide. Also, we have initiated rAd49 vector receptor usage suggesting that rAd49 utilizes hCD46 as a cellular receptor. Finally, the immunogenicity of the rAd49 vector was assessed and it was shown that an rAd49.SIVGag vaccine induces strong anti-SIVGag CD8+ T-lymphocytes in naïve mice, albeit less than an rAd5.SIVGag vaccine. However, in mice with high anti-Ad5 immunity the rAd5.SIVGag vaccine was severely blunted, whereas the anti-SIVGag response was not significantly suppressed using the rAd49.SIVGag vaccine. These data demonstrate the potential of a replication deficient human group D adenoviral vector for vaccination purposes.
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Introduction
The high prevalence of pre-existing immunity against adenovirus 5 (Ad5) may seriously limit the clinical utility and immunogenicity of rAd5-based vaccines. Therefore, many strategies are being developed to circumvent anti-Ad5 neutralizing activity without compromising yield, scale of manufacture or potency of antigen-specific immunity obtained with an rAd5 vector. We have previously reported on the successful generation of replication-incompetent vectors based on subgroup B-derived rare human serotype 11 (136) or 35 (344) and demonstrated that these vectors effectively by-pass anti-Ad5 immunity. Both the rAd11 and rAd35 vaccine carriers were generated with the aim to perform prime-boost vaccination, necessary to achieve long-term protection, since it has been amply shown that homologous prime-boost regimens using adenoviral vectors are unsuccessful due to anti-vector immunity induced upon vector priming (179, 241). While we found no evidence of immunological cross-neutralization between rAd11 and rAd35 vectors in human serum samples (136), subsequent detailed mouse immunization studies demonstrated that rAd11 and rAd35 are cross-reactive. This cross-reaction between rAd35-rAd11 vectors resulted in a less effective prime-boost regimen compared with combinations with rAd35-rAd5 or rAd11-rAd5 in naïve mice (179). However, these studies also revealed that in the presence of high anti-Ad5 neutralizing activity any prime-boost regimen using an rAd5 vaccine failed, leaving rAd35- rAd11 the best combination in such hosts (179). These results provided a rationale to search for a novel adenoviral vector, which is immunologically distinct from either rAd5 or rAd35. We have previously reported, using a limited number of human serum samples, that Ad49 displays low seroprevalence in Europe (344). Since Ad49 is a member of subgroup D it could therefore provide a superior priming or boosting vector. To obtain a replication- incompetent rAd49 vector, we generated a plasmid system that allows rAd49 genome manipulation. We demonstrate that E1-deleted rAd49 vectors can be produced to high titre on existing Ad5 E1-complementing cells such as PER.C6 (82) provided that the open reading frame (ORF) 6 derived from the adenoviral E4 region is replaced within the rAd49 backbone by the rAd5 E4-derived ORF6, as described recently for rAd11 and rAd35 group B vectors (123). Using caesium chloride-purified rAd49 luciferase batches, we performed extensive seroprevalence studies using serum samples derived from healthy volunteers in Europe, Asia, USA and Africa, showing that neutralizing activity towards Ad49 is rare worldwide. Initial tropism studies using rAd49.eGFP vector indicate that mouse cells (B16F10) expressing the BC1 isoform of hCD46 (343) are significantly better transduced by rAd49 as compared with parental B16F10 cells indicative of a role for hCD46 in cell entry. Finally, rAd49.SIVGag vaccination demonstrate that clear dose-dependent anti-SIVGag CD8+ T-lymphocyte responses can be obtained both in naïve mice and in mice carrying high anti-Ad5 immunity. Collectively, the data demonstrate that the novel rAd49 vaccine carrier can be produced to high titres on existing E1-complementing cell lines such as PER.C6, utilizes hCD46 as a receptor and elicits clear antigen-specific CD8+ T-lymphocyte responses in mice in the presence or absence of anti-Ad5 immunity.

Materials and Methods
Ad49 genome sequence
Wild-type (wt) Ad49 virus was propagated on PER.C6 cells and purified as described previously (344). The nucleotide sequence of wt Ad49 was determined via shotgun sequence technology (Lark Technologies) essentially as described previously (344). The 35 215 nt Ad49 genome sequence is publicly available under GenBank accession no DQ393829.

Primary cells and cell lines
PER.C6 (82) cells were routinely maintained in Dulbecco’s modified Eagle medium (DMEM; Gibco-BRL) supplemented with 10 % fetal bovine serum (FBS; Invitrogen) and 10 mM MgCl2. Hamster cell lines CHO-K1,
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Pro-5 and Lec2 (obtained from the ATCC) were cultured according to instructions provided. The CHO-hCAR cell line was maintained in RPMI supplemented with 10 % FBS. Mouse cell lines B16F10 and B16F10-hCD46 (343) were maintained in DMEM supplemented with 10 % FBS.

**Ad49 plasmid system and vector generation**

A similar construction strategy has been applied to generate E1-deleted Ad49 vector as described previously (136, 344). The plasmid system consisted of an adapter plasmid (pAdApt49) containing the left end of the Ad49 genome with deletion of E1 sequences and a cosmid (pWE.Ad49.E3.5ORF6) containing 31.5 kb of the Ad49 genome. Plasmid pAdApt49 (nt 1-462 of wt Ad49) contained the left inverted terminal repeat (ITR) and the packaging signal, encompassing an expression cassette consisting of the cytomegalovirus promoter linked to a multiple cloning site (MCS) followed by the simian virus 40 poly(A) transcription termination signal, and further containing part of the Ad49 wt genome corresponding to nt 3362-5909. This latter sequence enables homologous recombination with the Ad49 cosmid in Ad5 E1-complementing cells like PER.C6. Cosmid pWE.Ad49.E3.5ORF6 contained the Ad49 genome starting from pX to the right ITR (nt 3750-35 215), a 4 kb deletion in the E3 region and the Ad5 E4-ORF6 replacing the Ad49 E4-ORF6. To introduce the 4 kb deletion in the E3 region a smaller pBr-based subclone of Ad49, pBr.Ad49.SrfI-rITR, was generated that contained Ad49 nt 15 364-35 215. The pBr.Ad49.SrfI-rITR.E3 plasmid was thereafter constructed by generating two PCR fragments flanking the Ad49 E3 region (nt 25 461-26 665 and 30 736-33 488). Both PCR fragments contained an artificially introduced AflII restriction site to ligate both PCR fragments. The ligated PCR fragments were subsequently digested with AflII and EcoRI and ligated into an AflII-EcoRI digested pBr.Ad49.SrfI-rITR plasmid, thereby deleting 4 kb of the Ad49 E3 region (nt 26 666-30 735). The Ad49 E4-ORF6 was there-after exchanged by the Ad5 E4-ORF6, to enable growth of rAd49 on PER.C6 cells as described previously (123). Briefly, the sequence from nt 32 257 to 33 389 was replaced by the corresponding sequence of Ad5 by assembling three PCR fragments. The first PCR fragment consisted of 1.5 kb spanning nt 31 348-12 882 of pBr.Ad49.SrfI-rITR.E3 containing a 3’tail homologous to the Ad5 E4-ORF6/7 sequence. A second PCR fragment was obtained by using an Ad5 cosmid clone containing nt 3534 to the right ITR (pWE.Ad5.AflII-rITR, see (344)), resulting in a 1.1 kb fragment corresponding to Ad5 sequence nt. 32 963-34 077 (as in GenBank accession no. M73260) and flanked by sequences homologous to the first (3’end) and third (5’end) PCR fragments.

The third PCR fragment of 109 bp coding for the Ad49 E4-ORF6 nt 14 016-14 125 of pBr.Ad49.SrfI-rITR was obtained by using the pBr.Ad49.SrfI-I restriction enzyme to ligate both PCR fragments. The ligated PCR fragments were subsequently digested with AflII and EcoRI and ligated into a AflII-EcoRI digested pBr.Ad49.SrfI-rITR plasmid, thereby deleting 4 kb of the Ad49 E3 region (nt 26 666-30 735). The Ad49 E4-ORF6 was exchanged by the Ad5 E4-ORF6, to enable growth of rAd49 on PER.C6 cells as described previously (123). Briefly, the sequence from nt 32 257 to 33 389 was replaced by the corresponding sequence of Ad5 by assembling three PCR fragments. The first PCR fragment consisted of 1.5 kb spanning nt 31 348-12 882 of pBr.Ad49.SrfI-rITR.E3 containing a 3’tail homologous to the Ad5 E4-ORF6/7 sequence. A second PCR fragment was obtained by using an Ad5 cosmid clone containing nt 3534 to the right ITR (pWE.Ad5.AflII-rITR, see (344)), resulting in a 1.1 kb fragment corresponding to Ad5 sequence nt. 32 963-34 077 (as in GenBank accession no. M73260) and flanked by sequences homologous to the first (3’end) and third (5’end) PCR fragments.

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**Determination of neutralizing antibody titers**

To determine the NAb titres against wt Ad49 in mouse sera, the wt virus replication inhibition assay was used as described previously (344). To determine the NAb titres against Ad5 and rAd49, sera from mouse or human origin were heat inactivated for 1 h at 56°C, followed by the luciferase transgene inhibition detection assay as described previously (308). All human sera were obtained after informed consent.

**Ad49 tropism studies**

Cell lines were cultured in 24-well plates and exposed to 1000 virus particles (vp) per cell of rAd49.eGFP. Virus exposure was allowed for 2 h at 37°C, upon which medium was replaced. After 48 h, cells were harvested, washed with PBS/0.5 % BSA, centrifuged and resuspended in cell fix (Becton Dickinson) prior to fluorescence-activated cell sorter (FACS) analysis. To determine the NAb titres against rAd5 and rAd49, sera from mouse or human origin were heat inactivated for 1 h at 56°C, followed by the luciferase transgene inhibition detection assay as described previously (308). All human sera were obtained after informed consent.

**Vaccination experiments, SIVGag-specific tetramer-binding studies and ELISPOT**

Six- to 8-week-old C57BL/6 mice were obtained from Harlan B.V. (Zeist, The Netherlands). To induce anti-Ad49 or anti-Ad5 immunity, mice were pre-immunized twice, separated by a 4 week interval, with 10^7 vp replication-
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Competent Ad49 or 10^{10} vp replication-incompetent rAd5-Empty, respectively (total volume 100 µl in the quadriceps muscles). For immunization, mice received (intramuscularly, i.m.) 10^6, 10^7, 10^8, 10^9 or 10^{10} vp of replication-incompetent rAd5 or rAd49 (both E1/E3 deleted) expressing SIVGag. Tetrameric H-2D^d complexes folded around the immunodominant SIVGag AL11 epitope (AAVKNWMTQTL) (20) were prepared and utilized to stain peptide-specific CD8^+ T lymphocytes as described previously (6, 18). Samples were analyzed by two-colour flow cytometry on the FACScalibur (Becton Dickinson) and Cell-Quest Pro software. Gated CD8^+ T lymphocytes were examined for staining with the D^b/AL11 tetramer. SIVGag-specific cellular immune responses were assessed by gamma interferon (IFN-γ) ELISPOT assays as described previously (20, 316). Murine splenocytes were assessed for responses to individual SIVGag epitope peptides AL11 and KV9 (20) or a pool of overlapping 15 aa peptides covering the entire SIVmac239 Gag protein. Read out was done using an ELISPOT reader (Aelvis).

Statistical analyses
Tetramer, ELISPOT and cell infection data are shown as mean and standard error of the mean (SEM). Statistical analyses were performed using SPSS version 13. Comparison of tetramer responses between groups of mice were done for day 10 to 28 following immunization by ANOVA, since up to day 10 no change in response was either measured or anticipated. ELISPOT responses among groups of mice were compared by two-tailed t tests after logarithmic transformation. The percentages positive eGFP cells following cell infection were compared by ANOVA using the number of different experiments as co-factor. In all cases, P<0.05 was considered significant.

Results
Immunological cross-reaction between Ad49 and Ad5
To assess potential cross-reactive immunity between Ad5 and Ad49, groups of C57BL/6 mice (n=8 per group) were pre-immunized twice at -8 weeks and -4 weeks, with 10^{10} vp wt Ad49. Specific NAb anti-Ad49 titres in these mice reached a geometric mean titre (GMT) >6000. Subsequently, these mice were immunized once i.m. at day 0 with 10^9 vp rAd5.SIVGag. As shown in Fig. 1a, mean tetramer-positive CD8^+ T-lymphocyte responses from day 10 to 28 were not significantly different (P=0.13 for tetramer analysis using ANOVA tests) between naïve control mice and mice carrying high-level anti-Ad49 neutralizing activity. This observation was confirmed (P>0.05 comparing pooled peptide or peptide-specific ELISPOT responses using two-tailed t tests) by day 29 ELISPOT analyses against either pool or dominant and subdominant SIVGag epitopes (Fig. 1b). These data show that the rAd5.SIVGag vaccine was not hampered by high-level anti-Ad49 neutralizing activity, suggesting that cross-reaction between Ad49 and Ad5 might be minimal if any.

Figure 1: Interference of anti-Ad49 NAb with rAd5.SIVGag vaccine potency.
(a) SIVGag-specific CD8^+ T-lymphocyte responses were assessed by D^b/AL11 tetramer-binding assays at multiple time points following rAd5.SIVGag immunization of naïve (■) C57BL/6 mice (n = 8 per group) or mice pre-immunized twice with 10^{10} vp wt Ad49 (▲). (b) Day 29 IFN-γ ELISPOT assays on splenocytes derived from rAd5.SIVGag immunized, naïve (-) animals or Ad49-pre-immunized (+) animals using pooled SIVGag peptides (Gag) and individual dominant (AL11) and subdominant (KV9) epitope peptides. The means ± SEM for each data point are shown.

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Generation of a ΔE1-rAd49 vector plasmid system

Based on the genome sequence, the overall organization and location of ORFs within Ad49 prove very similar to other subgroup D members, including an extra ORF in the E3 region encoding 49K (73) (data not shown). The overall DNA similarity of Ad49 with Ad5 proved low (56%) and striking differences on an amino acid level are found in structural capsid proteins, hexon and fiber. For the hexon capsid protein, which determines the antigenicity of adenovirus (331, 364), the similarity within the hypervariable region (HVR) reached only up to 60% between Ad49 and Ad5. All regions of the fiber capsid, which is involved in cellular attachment (33, 96, 275) showed low similarity to Ad5, 58, 38 and 52% for tail, shaft and knob region respectively (Fig. 2a). In order to generate replication-incompetent rAd49 vectors a two-plasmid system was designed as shown schematically in Fig. 2b. Transfection of the two plasmids into the Ad5 E1-complementing PER.C6 cells did not result in progeny virus, demonstrating that the E1 region derived from Ad5 present in PER.C6 cells does not trans-complement for the E1 deficiency within the rAd49 genome (data not shown). However, as described recently for rAd11 and rAd35 vectors (123), E1-deleted rAd49 vector could be propagated successfully to high titers on PER.C6 when the Ad5 derived E4-ORF6 was cloned into the rAd49 backbone thereby replacing the native Ad49 E4-ORF6. To obtain purified research batches of E1-deleted rAd49 vector, production and purification protocols used for the manufacture of rAd5 vectors could be directly used, resulting in post-purification yields on research scale of 1 x 10^{12} vp ml^{−1} on average (n = 10).

![Figure 2: Ad49 genome organization and rAd49 vector plasmid system.](image)

(a) Schematic representation of the genome organization of wt Ad49 showing locations of the early genes (E), late genes (L), inverted terminal repeats (ITR) and packaging signal (ψ). Also shown are the amino acid similarity levels (indicated by the numbers) between Ad49 and Ad5 within hexon constant, hypervariable region (HVR) and the fiber region. (b) Schematic overview of the rAd49 plasmid system generated, which allows formation of rAd49 vector progeny on PER.C6 cells (see text for details).

Human seroprevalence and rAd49 titration studies

Human sera, obtained from healthy blood donors (20-70 years of age) derived from Japan (Tokyo, n=100), the USA [Stanford, California (n=100) and Great Neck, New York State, n=100]), the UK (n=100) and sub-Saharan Africa (n=200), were used to determine the seroprevalence of serotype Ad49 and Ad5. The data obtained demonstrate that Ad49 is
much less seroprevalent (ranging from 6 to 22 %) as compared with Ad5 (ranging from 48 to 98 %) in all geographical locations investigated (Fig. 3a). Also, the titres against Ad49 proved significantly less high (on average >10-fold) as compared with Ad5 (Fig. 3b). These data extent our previous limited Ad49 seroprevalence data in Europe using a wt virus replication inhibition assay (344). Next, we assessed whether there is an association between Ad5 and Ad49 seroprevalence by determining the odds ratio (Fig. 3c). Similar proportions of Ad49 seroprevalence were found among those tested positive for Ad5 as compared with those tested negative for Ad5. Therefore, no association was found for the double-positive sera between Ad5 and Ad49 NAb [odds ratio = 1.8 (95% confidence interval 0.8-3.8), \( P=0.13 \)]. Also when different regions were considered the odds ratio did not change. These data further indicate the absence of cross-reactive Ad49/Ad5-specific immunity.

Receptor usage of rAd49 vector

Since studies to date revealed a striking diversity in receptor usage of human group D adenoviruses including CAR, CD46 and \( \alpha(2\rightarrow3) \) sialic acid (13, 14, 274, 275, 366), we tested a panel of cell lines either positive or negative for these entry molecules. Hereto, we tested the transduction efficiency of rAd49.eGFP vector in Lec2 and Pro-5 cells that are negative or positive for \( \alpha(2\rightarrow3) \) sialic acid residues, respectively (Fig. 4a and ref (309)). As shown in Fig. 4b, no significant increase in either percentage eGFP-positive cells (40 vs 39% for Lec2 and Pro-5, respectively, \( P=0.6 \) ANOVA tests) or median fluorescence (data not shown) was observed between Lec2 and Pro-5, indicating that \( \alpha(2\rightarrow3) \) sialic acid expression does not contribute to rAd49 transduction. Identical experiments, performed
using CHO or CHO cell lines expressing the human coxsackie B adenovirus receptor (hCAR) (Fig. 4c), also demonstrated that hCAR expression (Fig. 4d) does not increase rAd49-mediated eGFP expression (36% for CHO-hCAR and 48% for CHO). Finally, we tested B16F10 cells and B16F10 cells expressing hCD46 (Fig. 4e), demonstrating that rAd49-mediated transduction is significantly ($P<0.001$ ANOVA tests) higher in B16F10-hCD46 (51%) as compared with B16F10 (15%), indicating that the BC1 isoform of hCD46 provides a cellular receptor for rAd49 (Fig. 4f). However, rAd49 vector transduced between 40 and 51% of Pro-5, Lec2, CHO cells as compared with low transduction efficiency using B16F10 mouse cells. This phenomenon could indicate that, besides CD46, rAd49 can recognize another receptor expressed on hamster cells and not on mouse cells.

**Figure 4:** Receptor usage by rAd49 vector.
FACS histograms showing $\alpha$(2→3) sialic acid expression in Pro-5 and Lec2 cells used (a) and percentage of eGFP-positive Pro-5 (shaded bar) or Lec2 cells (empty bar) obtained 48 h after rAd49 vector exposure (b). hCAR expression in CHO and CHO-hCAR cell lines used (c) and percentage of eGFP-positive CHO (shaded bar) or CHO-hCAR cells (empty bar) obtained 48 h after rAd49 vector exposure (d). hCD46 expression on B16F10 and B16F10-hCD46 cells used (e) and percentage of eGFP-positive B16F10 (shaded bar) or B16F10-hCD46 cells (empty bar) obtained 48 h after rAd49 vector exposure (f). In all cases, cells were exposed for 2 h to 1000 vp per cell.

*rAd49.SIVGag* vaccine potency in presence and absence of anti-Ad5 immunity
To investigate the ability of rAd49 vector to elicit antigen-specific immune responses, we generated an rAd49 vector carrying the SIVGag antigen. Groups of C57BL/6 mice ($n=8$ per group) were immunized once i.m. with $10^{10}$, $10^9$, $10^8$, $10^7$ or $10^6$ vp of rAd49.SIVGag vaccine or rAd5.SIVGag as control. As shown in Fig. 5a, potent SIVGag-specific CD8$^+$ T-lymphocyte responses were obtained at vaccine doses beyond $10^6$ vp for rAd5 with peak responses at $10^6$ vp. Upon immunization with rAd49 vaccine SIVGag-specific CD8$^+$ T-lymphocyte responses were also clearly detected at doses exceeding $10^5$ vp (Fig. 5b),
although responses were significantly lower (for dosage $10^9$ vp, $P<0.001$ for tetramer analysis day 10 to 28, using ANOVA tests) as compared with rAd5.SIVGag vaccine and peaked at the $10^{10}$ vp doses. The higher immunogenicity in naïve C57BL/6 mice of an rAd5 vaccine as compared with an rAd49 was confirmed (Fig. 5c and d, respectively) via ELISPOT assays ($P<0.05$ comparing pooled peptide or peptide-specific ELISPOT responses using two-tailed $t$ tests).

**Figure 5:** Immunogenicity of rAd5.SIVGag or rAd49.SIVGag vectors in naïve mice.
SIVGag-specific CD8$^+$ T-lymphocyte responses were assessed by D$b$/AL11 tetramer-binding assays at multiple time points following immunization of naïve C57BL/6 mice ($n=8$ per group) with $10^5$, $10^6$, $10^7$ or $10^8$ vp rAd5.SIVGag (a) or rAd49.SIVGag vaccine (b). At day 29, IFN-γ ELISPOT assays were performed on splenocytes derived from rAd5.SIVGag (c) or rAd49.SIVGag (d) vaccinated animals using pooled SIVgag peptides (Gag) or dominant (AL11) and subdominant (KV9) epitope peptides. The mean ± SEM response for each data point is shown.

Next, we assessed the impact of anti-Ad5 vector immunity on the immunogenicity of an rAd49.SIVGag vaccine. Hereto, C57BL/6 mice ($n=8$ per group) were pre-immunized twice, at week -8 and week -4, with $10^{10}$ vp rAd5.Empty vector to artificially induce anti-Ad5 vector immunity. This pre-immunization regimen resulted in Ad5-specific NAb titres above the level normally seen in human individuals from either developed or developing world (174), with a GMT beyond 32 768. At day 0, mice were immunized i.m. with $10^9$, $10^8$, $10^7$ or $10^6$ vp of rAd49.SIVGag or rAd5.SIVGag as control. As shown in Fig. 6a, the anti-SIVgag CD8$^+$ T-lymphocyte response was severely ablated upon vaccination with rAd5.SIVGag when compared with the anti-SIVGag CD8$^+$ T-lymphocyte response obtained in naïve mice (Fig. 5a) ($P<0.001$ for tetramer analysis day 10 to 28, using ANOVA tests and comparing pooled peptide or peptide-specific ELISPOT responses using two-tailed $t$ tests), which was also observed in the ELISPOT assay (Fig. 6c in comparison with Fig. 5c). In contrast, immunization with rAd49.SIVGag vaccine demonstrated potent CD8$^+$ T-
lymphocyte responses as observed using tetramer staining and ELISPOT analyses (Fig. 6b and d, respectively). Comparison of CD8⁺ T-lymphocyte responses upon rAd49.SIVgag immunization in either naïve mice or mice carrying high level anti-Ad5 neutralizing activity demonstrated that presence of anti-Ad5 NAb did not interfere with rAd49 vaccine potency beyond 10⁹ vp (P>0.05 for tetramer analysis day 10 to 28, using ANOVA tests and P>0.05 comparing pooled peptide ELISPOT responses using two-tailed t tests).

Collectively, these data show that rAd49 proves less immunogenic in naïve C57BL/6 mice as compared with rAd5 but in presence of anti-Ad5 pre-existing immunity the potency of the rAd49 vaccine is not ablated as compared with rAd5.

**Figure 6:** Immunogenicity of rAd5.SIVGag or rAd49.SIVGag vectors in mice carrying high anti-Ad5 immunity.

C57BL/6 mice were pre-immunized twice, separated by a 4 week interval, with 10⁹ vp replication-competent Ad5.Empty before immunization. SIVGag-specific CD8⁺ T-lymphocyte responses were assessed by D¹/AL11 tetramer-binding assays at multiple time points following immunization of the Ad5 pre-immunized mice (n = 8 per group) with 10⁵, 10⁶, 10⁷ or 10⁸ vp rAd5.SIVGag (a) or rAd49.SIVgag vaccine (b). At day 29, IFN-γ ELISPOT assays were performed on splenocytes derived from rAd5.SIVGag- (c) or rAd49.SIVgag- (d) vaccinated animals using pooled SIVGag peptides (Gag) or dominant (AL11) and subdominant (KV9) epitope peptides. The mean ± SEM response for each data point is shown.

**Discussion**

We have previously developed adenoviral vectors derived from rare human serotypes such as rAd11 (136) and rAd35 (344) and have demonstrated that vaccines based on these carriers are not hampered by the wide-spread and high-titred anti-Ad5 immunity, in contrast to rAd5. Although both rAd11 and rAd35 were successful in inducing potent antigen-specific immune responses (20, 179), prime-boost regimens with these vectors revealed immunological cross-reaction leading to less effective vaccination as compared with rAd35-rAd5 or rAd11-rAd5 regimens in naïve mice. Further investigation by both pre-immunization- and adoptive-transfer studies indicated that neutralizing antibodies, as well as
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CD8+ T lymphocytes (179), contribute to the observed cross-reaction between rAd11 and rAd35. Based on these observations a vector, which is immunologically distinct from both rAd5 and rAd11 or rAd35, could provide a superior vector in prime-boost applications.

In an earlier survey using 100 human serum samples from healthy individuals in Europe, Ad49 demonstrated low seroprevalence with <10% positive sera (344). Due to the low seroprevalence observed in this limited screen against Ad49 and since Ad49, being a subgroup D vector, should be immunologically distinct from rAd5 and rAd11 or rAd35, we chose to develop this virus into a vector system.

The genome sequence of Ad49 revealed the general adenoviral genome organization with well-defined early and late gene locations. ORFs proved very similar to other subgroup D members including an extra ORF in the E3 region encoding 49K (73). The function of the subgroup D-specific E3/49K protein is not known; however, the sequence predicts it to be a type-I transmembrane protein (360). Structural proteins important for virus assembly (100K, pIIa, pV, pVII and pX) are highly conserved (99-100 %) between Ad49 and two other known subgroup D adenoviruses, i.e. Ad9 and Ad46. The similarity of the ORF6 protein derived from the adenoviral E4 region proved to be low between Ad49 and Ad5 (59%). This possibly helps to explain the observed lack of rAd49 vector propagation on PER.C6 cells due to a poor interaction between the Ad5-derived E1 proteins and the Ad49-derived ORF6 protein. The latter interaction is pivotal, since a complex between the E1B-55K protein and the E4-ORF6 protein (109) mediates viral mRNA transport necessary for late viral gene expression and virus replication (354).

Using replication-incompetent rAd49 vector carrying the luciferase marker gene, we evaluated the worldwide seroprevalence of Ad49 and demonstrated overall low pre-existing immunity and serum titres towards Ad49, including sub-Saharan Africa. At present it is not well understood why on all continents seroprevalence against Ad49 is detected, indicative of global viral spread, whereas antibody titers against Ad49 are generally low. The first Ad49 isolation was reported in 1993 (292) and most Ad49 isolations have been reported from AIDS patients, like most human adenoviruses (62, 130, 131, 292). However, it has been reported that Ad49 infections are not strictly limited to HIV-positive individuals, since Ad49 could also be isolated from clinical samples obtained from apparently healthy individuals, though rarely (62, 68). These findings, coupled to the observed overall low pre-existing immunity towards Ad49 could indicate that Ad49 has either difficulty to establish productive infection due to, for instance, impaired transmission, or that Ad49 has developed an immune evasion strategy to escape host immune responses. Although further studies are needed, we attribute the observed low anti-Ad49 pre-existing immunity to impaired transmission, based on observations that human individuals can elicit potent antibody responses against Ad49 and that apparently Ad49 is well controlled in healthy individuals since Ad49 is predominantly isolated from immune compromised individuals. Also, our experiments in mice demonstrate that upon exposure to wt Ad49 these animals elicit high-titred anti-Ad49 antibody levels.

Next, we initiated studies to assess the receptor usage of our rAd49 vector. Studies to date have revealed a striking diversity in receptor usage of human group D adenoviruses (13, 14, 274, 275, 366), which prompted us to initiate studies upon rAd49 receptor usage. Our data indicate that rAd49 seems to utilize hCD46, whereas evidence for a role of either sialic acids or CAR could not be established. Analyses of the Ad49 fiber protein sequences revealed that, although our rAd49 does not seem to utilize CAR, the most important CAR-binding residues are present (35, 163, 276), a phenomenon previously reported for Ad37. For Ad37, it was shown that this serotype has the ability to bind to the CAR protein but binding did not result in virus entry (365). The authors attributed the inability of cell entry to the short Ad37 fiber shaft, making the fiber too rigid to allow infection (365). This finding supported an
earlier study showing that an Ad5 vector carrying the fiber shaft of Ad9 had a reduced CAR-binding and cellular-infection capability (298). Like Ad37, our rAd49 vector has a short fiber shaft, consisting of only eight repeats, which might indicate that Ad49 perhaps can bind CAR but cannot enter cells, although further studies are needed to investigate this hypothesis.

Within the rAd49 fiber sequence two of the five possible key residues required for sialic acid binding (43), Tyr312 and Lys345, are present, without finding evidence in our studies of productive rAd49 infection through sialic acid binding. This is indicative for an important role of the other three residues (43). For subgroup B adenoviruses, as well as for subgroup D member Ad37, it has recently been shown that they can use hCD46 as a cellular receptor (96, 293, 305, 366) and our studies indicate that Ad49 is also able to use hCD46. Although at present it is unknown whether all hCD46 isoforms (C1, C2, BC1 and BC2) can be utilized by rAd49, our data shows that at least the BC1 isoform is used.

We next assessed the potency of an rAd49.SIVGag vaccine in naïve mice, demonstrating that strong SIVGag-specific CD8+ T-lymphocyte responses are elicited, although less high than rAd5 based vaccine. The insert-specific immunity induced by an rAd49 vaccine seemed similar to the anti-SIVGag immune induction obtained with an rAd35 and rAd11 vaccine in naïve mice (20, 179). This lower immunogenicity might be explained by the fact that all these vectors use hCD46 as a cellular receptor, which is absent in inbred strains of mice (343), in contrast to the CAR receptor utilized by rAd5. The value of rAd49 as vaccine carrier was subsequently demonstrated in experiments showing that SIVGag responses were not impaired in hosts carrying high anti-Ad5 neutralizing activity, whereas rAd5-mediated SIVGag responses were blunted in such hosts.

Collectively, the data demonstrate that replication-incompetent rAd49 vectors can be efficiently made on established Ad5 E1-complementing cell lines such as PER.C6, resulting in high titre vector batches. Initial studies regarding tropism indicate that the rAd49 vector utilizes hCD46 as receptor and vaccination studies with an rAd49 vector demonstrate that this vaccine carrier induces insert-specific CD8+ T-lymphocyte responses in either presence or absence of anti-Ad5 immunity. Future prime-boost studies in combination with the previously developed rare human adenoviral vectors will indicate whether an optimal heterologous adenoviral prime-boost regimen can be established in presence of high anti-Ad5 pre-existing immunity.

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