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ORIGINAL ARTICLE

Chimpanzee-origin adenovirus vectors as vaccine carriers

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Vaccines based on replication-defective adenoviral vectors are being developed for infectious agents and tumor-associated antigens. Early work focused on vaccines derived from a common human serotype of adenovirus, that is, adenovirus of the serotype 5 (AdHu5). Neutralizing antibodies against AdHu5 virus, present in a large percentage of the human population, dampen the efficacy of vaccines based on this carrier. To circumvent this problem, we generated vectors derived from chimpanzee adenoviruses. Here we describe some basic parameters of vectors derived from chimpanzee adenoviruses C68 and C7, including growth characteristics, yields of infectious particles, effects of additional deletions in E3 and E4 and lengths of the inserted foreign sequence as they relate to the suitability for their eventual development as vaccine carriers for clinical use.
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Introduction

Recombinant adenoviruses (Ad), originally used as vehicles for gene replacement therapy,[1] have become favorite vaccine carriers for delivery of genes derived from pathogens[2–4] or tumors.[5] They elicit strong and sustained transgene product-specific immune responses upon delivery of a single dose given either systemically[6,7] or via mucosal routes.[8] On a practical note Ad vectors developed to date accommodate comparatively large pieces of foreign DNA, they can be grown to high titer in available cell lines, and methods for purification and scale-up production are well established, which facilitates their development for clinical use. Most of the past efforts focused on human Ads, such as those of the common serotype 5 (AdHu5), which cause mild upper respiratory symptoms upon natural infections. Both replication competent[9,10] and E1-deleted (ΔE1) replication-defective AdHu5 vectors have been developed as vaccine carriers and have shown efficacy in mice,[11] dogs[12,13] and nonhuman primates (NHPs).[7] E1-deleted AdHu5 vectors also performed well in clinical trials.[14]

A substantial portion of human adults carries virus-neutralizing antibodies to AdHu5 virus due to previous natural infection. In the US about 45% of the adult human population has significant titers of virus neutralizing antibodies to AdHu5 virus[15] and this percentage is comparable in Africa and even higher in Asia. As was demonstrated in experimental animals and subsequently in human volunteers, virus-neutralizing antibodies (VNAs) to AdHu5 virus reduce the potency of AdHu5-based vaccines.[6,16] VNAs block cell entry by Ad vectors, which is a prerequisite for synthesis of the transgene product. We addressed the critical problem of pre-existing immunity on the efficacy of Ad-based vaccine carriers by developing vectors derived from chimpanzee-origin Ad viruses termed AdC68 and AdC7 or Pan9 and Pan7, respectively.[15–17] These vaccine carriers are based on molecular clones[18] thereby circumventing risks associated with potential contamination of the vaccines with unidentified pathogens of simian origin. AdC68 and AdC7 do not circulate in the human population.[15] VNAs to AdC68 partially neutralize AdC7 and vice versa. Both viruses are closely related to human serotype 4 Ad.[15] AdC68 and AdC7 vectors, similar to AdHu5 vectors, induce potent transgene product-specific $CD8^+$ T-cell and B-cell responses.[6,16] Transgene product-specific B-cell responses elicited by the chimpanzee-origin vectors are not impaired by pre-existing immunity to common human serotypes of adenovirus.[16] $CD8^+$ T-cell responses are slightly reduced and this reduction seems to be caused by $CD8^+$ T cells directed to conserved sequences of Ad.[6] Similar to AdHu5 virus, AdC7 and AdC68 viruses gain entry into cells through the coxsackie adenovirus receptor (CAR) and thus are expected to have a similar tropism to the well-studied AdHu5 virus.[19]

For clinical development, reproducible production of sufficient amounts of high-quality vectors that are genetically stable, show acceptable batch reproducibility and, in case of ΔE1Ad vectors, lack contamination with replication competent adenovirus (RCA), arising upon recombination of the vectors with the E1 gene present in the packaging cell line, is crucial. ΔE1AdC68 and ΔE1AdC7 vectors are *trans*-complemented by the E1 of AdHu5 virus.[15] The E1-flanking sequences differ

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between AdHu5 and the simian-origin viruses, which prevents homologous recombination and outgrowth of RCAs in ΔE1AdC68 or ΔE1AdC7 vector batches. For expression of large transgenes, deletions in addition to E1 such as of E3, which encodes immunoevasion polypeptides[20] that are nonessential for viral growth or E4, an essential region that has to be *trans*-complemented by the packaging cell line,[21] are needed. Such deletions may affect vector performance.

Here, we tested parameters of replication-defective AdC68 and AdC7 vectors that may affect their performance as vaccine carriers. Specifically we tested for batch variation in vector infectivity and how this affects the vectors' ability to induce transgene product-specific $CD8^+$ T-cell responses. We also assessed the effect of different deletions and variations in insert length on vector performance. Lastly, we compared SIV-1 antigen-specific $CD8^+$ T-cell responses induced by a polypeptide vaccine expressing a SIV-gag–pol–nef fusion protein to those elicited by a vaccine incorporating a mixture of adenovirus vectors individually expressing SIV-1 gag, pol and nef.

Results

Vector yield, contamination with RCA, infectivity and batch reproducibility

Moving experimental vaccine constructs into the clinic requires that they can be readily propagated to levels that allow for eventual large-scale production. In the case of ΔE1Ad vectors, lack of contamination with RCAs (arising from homologous recombination with the E1 of the packaging cell line) is also essential. Different packaging cell lines are available for propagation of ΔE1Ad vectors. HEK 293 cells carry the E1 gene of AdHu5 virus and, in addition, E1 flanking sequences,[22] which promote homologous recombination with ΔE1Ad-Hu5 vectors. PerC6 cells,[23] which are not readily available, only carry the E1 gene of AdHu5 virus, which considerably lessens the likelihood of E1 replacement in replication-defective AdHu5 vectors. Using vectors deleted in E1 and E4 can reduce outgrowth of RCAs of AdHu5 vectors on HEK 293 cells. The latter is essential for virus replication and has thus to be provided in trans by the packaging cell line. For our studies, ΔE1, ΔE1/E3 and ΔE1/E3/E4AdHu5, AdC68 and AdC7 simian vectors were propagated on HEK 293 cells carrying E1 or E1 and E4 of AdHu5 virus.

Vectors, their deletions, transgene products and names used throughout this study are listed in Table 1. Yields of large batches of vectors from simian serotype with different deletions expressing reporter proteins of viral antigens were determined by recovery of viral genomes (vp) from $\sim 10^9$ HEK 293 cells. They were compared to those of ΔE1E3AdHu5 vectors and to a modest number of ΔE1AdHu5 vectors. ΔE1AdC68 vectors depending on the transgene product in general gave slightly higher yields and showed more rapid plaque formation compared to AdHu5 vectors. Yields of ΔE1AdE7 vectors

Table 1 Description of adenovirus

Vector	*Deletions*[a]	*Inserts (length*[a]*)*	*Name of vector*
AdHu5	E1: 342–3529	HIVgag37 [1089]	ΔE1AdHu5HIVgag37
AdHu5	E1: 342–3529 E3: 27 865–30 995	HIV-1gag37 [1089]	ΔE1/E3AdHu5HIVgag37
AdHu5	E1: 342–3529 E3: 27 865–30 995 E4: 33 105–35 506	HIV-1gag37 [1089]	ΔE1/E3/E4AdHu5HIVgag37
AdC68	E1: 456–3014	HIV-1gag37 [1089] HIV-1gag [1503] $SIVmac_{239}$gag [1567] $SIVmac_{239}$nef [792]	ΔE1AdC68HIVgag37 ΔE1AdC68HIVgag ΔE1AdC68SIVgag ΔE1AdC68SIVnef
AdC68	E1: 456–3014 E3: 27 816–31 333	HIV-1gag37 [1089] HIV-1gag37+stuffer [3589] HIV-1 5′pol [1950] HIV-1 TPAnef-3′pol [1833] HIV-1gp140 [1924] $SIVmac_{239}$pol [2891] $SIVmac_{239}$gag–pol [4394] $SIVmac_{239}$gag–pol–nef [5277]	ΔE1/E3AdC68HIVgag37 ΔE1/E3AdC68HIVgag37+S ΔE1/E3AdC68HIV5′pol ΔE1/E3AdC68HIVTPAnef-3′pol ΔE1/E3AdC68HIVgp140 ΔE1/E3AdC68SIVpol ΔE1/E3AdC68SIVgag–pol ΔE1/E3AdC68SIVgag–pol–nef
AdC68	E1: 456–3014 E3: 27 816–31 333 E4: 33 737–36 277	HIV-1gag37 [1089]	ΔE1/E3/E4AdC68HIVgag37
AdC7	E1: 456–3014	HIV-1gag37 [1089]	ΔE1AdC7HIVgag37
AdC7	E1: 455–3029 E3: 27 775–31 299	$SIVmac_{239}$gag–pol [4394]	ΔE1/E3C7SIVgag–pol

[a]Deletions and transgene lengths are shown in bp.

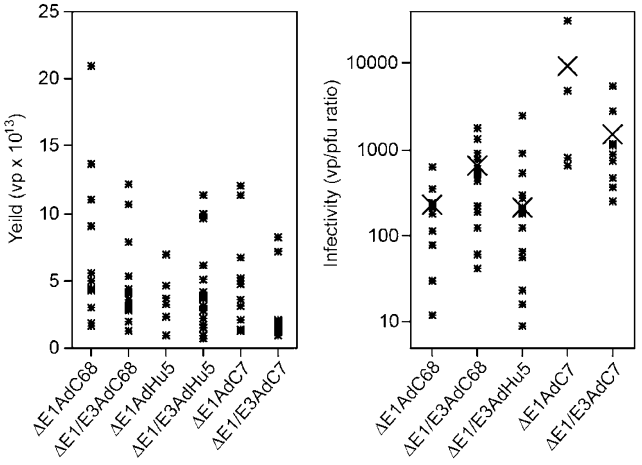

Figure 1 (**a**) Vector yield. The figure lists the yields obtained from vectors grown on 50 plates (15 cm Petri dishes) of HEK 293 cells (ΔE1AdC68: $n=14$; ΔE1/E3AdC68: $n=17$; ΔE1AdHu5: $n=6$; ΔE1/E3AdHu5: $n=16$; ΔE1AdC7: $n=11$; ΔE1/E3AdC7: $n=11$). Vectors expressed a variety of reporter proteins or viral antigens. The *Y*-axis shows yield $\times 10^{13}$ virus particles. (**b**) Infectivity of vectors. The graph shows the ratios of vp to PFU of individual vector batches. The X symbols indicate geometric means (ΔE1AdC68: $n=10$; ΔE1/E3AdC68: $n=14$; ΔE1/E3AdHu5: $n=16$; ΔE1AdC7 $n=4$; ΔE1/E3AdC7: $n=9$).

Table 2 Comparison of inserted transgene size on yield and infectivity

Size of transgene	*Yield ($\times 10^{13}$ vps) ± s.d.*	*Infectivity (vp:PFU) ± s.d.*
<1600 bp	7.47 ± 5.55 (2.7–21.1)	387 ± 473 (36–1731)
>4300 bp	4.08 ± 2.69 (0.34–11.0)	603 ± 714 (41–2486)

The table shows the arithmetic mean ± standard deviations (and the range in parenthesis) for vector yields and vp-to-PFU ratios for E1 or E1 and E3 deleted AdC68 vectors. Vectors carried transgenes ranging in size from ~300 to 1600 bps (E7 of HPV-16, glycoprotein gene of rabies virus, gag of HIV-1 and SIV, $n=9$) or from ~4400–5300 bps (SIV or HIV-1gag–pol or gag–pol–nef fusion genes, $n=10$). Statistical significance for differences in yields and infectivity were calculated by Student's *t*-test (*P*-value for yields: 0.077, *P*-value for infectivity: 0.31).

were comparable to those of ΔE1AdHu5 vectors while ΔE1/E3AdC7 vectors commonly gave slightly lower yields. Overall, growth characteristics of the simian vectors on HEK 293 cells are favorable for scale-up production. None of the batches of simian vectors generated thus far showed contamination with RCAs. Vector infectivity measured by plaque assays dosed according to copy numbers of vector genome were roughly comparable between ΔE1AdC68, ΔE1/E3AdHu5 and ΔE1/E3AdC68 vectors while ΔE1AdC7 and ΔE1/E3AdC7 vectors commonly showed higher ratios of vp to plaque forming units (PFU) (Figure 1). To determine if the insert length affected vector yields or infectivity of the vector preparations, we compared 9–10 AdC68 vectors carrying small transgenes ranging in size from 300 to 1600 bp to those carrying large inserts of 4500 to 5000 bp. Although as shown in Table 2 vectors with small inserts tended to give higher yield and lower vp-to-PFU ratios, the differences were statistically not significant.

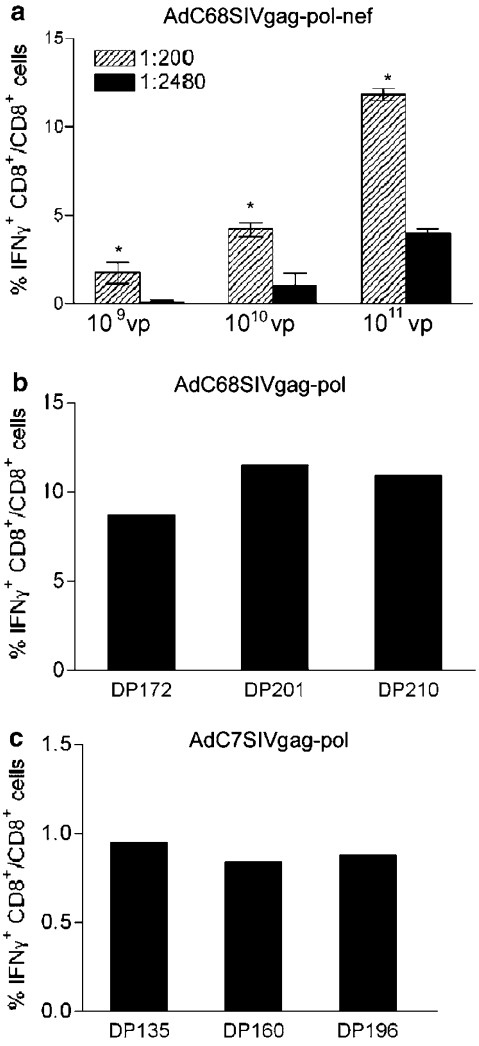

Figure 2 (**a**) Vector infectivity. C57Bl/6 mice (three per group) were immunized with escalating doses of ΔE1/E3AdC68SIVgag–pol–nef vectors from two vector batches that had different PFU:vp ratios (1:200 – striped bars, 1:2480 – closed bars). After 10 days, frequencies of gag-specific $CD8^+$ T cells in the spleen were determined by ICS. Results shown represent two independent experiments. *Indicates *P*-value <0.05 comparing frequencies elicited by vectors from the two preparations used at the same vector dose. (**b**, **c**) Vector reproducibility. C57Bl/6 mice (three per group) were immunized with one dose of ΔE1/E3AdC68SIVgag–pol (**b**) and ΔE1/E3AdC7SIVgag–pol (**c**) vectors. After 10 days, gag-specific $CD8^+$ T-cell frequencies were determined from splenocytes by ICS. Results shown represent two independent experiments.

Stimulation of transgene product-specific $CD8^+$ T cells is driven by peptides derived from *de novo* synthesized protein, requiring infectivity of the vector. To address the role of relative vector infectivity, we compared ΔE1/E3AdC68SIVgag–pol–nef vector batches with a vp-to-PFU ratio of 1:200 to a batch with a high vp-to-PFU ratio of 1:2480. C57Bl/6 mice were immunized intramuscularly with graded doses of vector and frequencies of splenic $CD8^+$ T cells to the immunodominant epitope of gag were measured 10 days later by intracellular cytokine staining (ICS). As shown in Figure 2a, vector infectivity influenced induction of transgene product-specific $CD8^+$ T cells. Approximately 10 times as much of the ΔE1/E3AdC68SIVgag–pol–nef vector batch, that

showed low infectivity, was needed to achieve by day 10 frequencies of gag-specific CD8+ T cells comparable to those induced by the batch that had an infectivity to vp ratio of 1:200. As toxicity of replication-defective Ad vectors, which is primarily mediated by proinflammatory cytokines released rapidly in response to the capsid proteins of Ad, is linked to vp rather than infectious units, the tendency of the AdC7 vectors to have lower yields of infectious particles may be of concern for their eventual clinical development. Nevertheless, it should be pointed out that most vector batches had ranges of vp to PFU (Figure 1) that did not affect induction of transgene product-specific CD8+ T cells.

To assess reproducibility of vector performances (a prerequisite for their use as vaccine carriers), three different batches derived independently from the same stock of ΔE1/E3AdC68SIVgag–pol vectors were tested. Comparable frequencies of gag epitope-specific CD8+ T cells were achieved (Figure 2b**)**. A parallel experiment using ΔE1/E3AdC7SIVgag–pol vectors yielded similar results (Figure 2c). It should be pointed out that vectors used for these experiments had vp-to-PFU ratios that showed at most a four-fold difference.

Effects of deletions of adenoviral genes on vector performance

For complex pathogens, such as HIV-1, which shows a high degree of sequence variability between different isolates and rapidly mutates upon infection of an individual, a vaccine has to induce an immune response of sufficient breadth. This requires induction of T cells to several viral antigens to allow for protection against wild-type strains of virus and to limit outgrowth of escape mutants upon infection. Accommodation of large sequences such as those of multiple antigens either as fusion proteins or as individual antigens into the genome of Ad vectors requires deletions in addition to E1. ΔE1Ad vectors accommodate ~3.5 kb of sequence, ΔE1/E3Ad vectors can carry ~7 kb of foreign sequence and further deletion of E4 increases the permitted insert size by an additional 3 kb. The effects of these different deletions have been studied in depth for AdHu5 vectors, but their impact on gene transfer by simian Ad vectors remains unclear. To determine whether additional deletions of the simian Ad backbone influence levels of transgene product expression, we transduced CHO cells stably expressing CAR with simian Ad vectors expressing a humanized, C-terminus truncated form of HIV gag (termed gag37)[24] under the control of the early promoter of cytomegalovirus. Ad vectors used in these experiments were deleted in E1 alone, E1 and E3 or in E1, E3, and E4 (Figure 3a). As shown, the E3 deletion did not have an effect on transgene product production while deletion of E4 decreased protein expression from both AdHu5 and AdC68 vectors. CD8+ T-cell responses to gag were also reduced when the E4-deleted vectors were used to immunize BALB/c mice (Figure 3b and c). The reduction in T-cell frequencies was most pronounced at the lower doses. Vectors deleted in E4 cannot be tested for plaque formation on HEK 293 cells and the poor performance of such vectors may in part reflect low contents of infectious particles. Alternatively, physiological functions of E4 polypeptides (which even in E1-deleted vectors are expected to be produced at

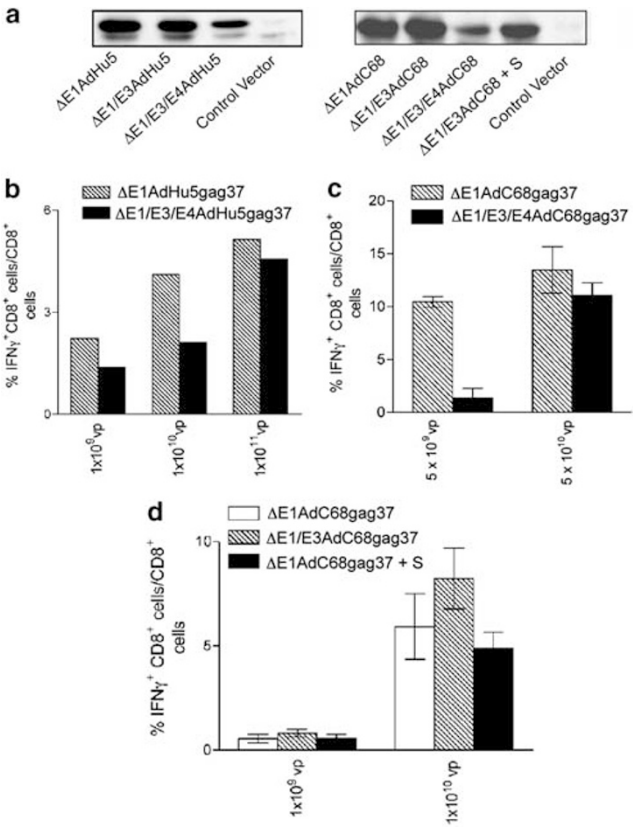

Figure 3 Early Ad gene deletions. (**a**) Ad backbone deletions can alter transgene product expression. CHO/CAR cells were infected with 1000 vp/cell of the indicated vectors. After 36 h, cells were harvested, lysed and an equal amount of total protein was used for anti-HIV-1 gag detection. (**b–d**) BALB/c mice (five per group) were immunized with graded doses of (**b**) ΔE1AdHu5HIVgag37, ΔE1/E3/E4AdHu5HIVgag37, (**c**) ΔE1AdC68HIVgag37, ΔE1/E3/E4AdC68HIVgag37, (**d**) ΔE1AdC68HIVgag37, ΔE1/E3AdC685HIVgag37, ΔE1/E3AdC68HIVgag37+S. Frequencies of gag-specific CD8+ T cells were determined by ICS 10 days later from spleens of individual mice (except for the AdHu5 experiment in panel b where spleens were pooled). Results shown represent two independent experiments.

albeit only low levels) may facilitate adaptive immune responses to the transgene product.

The consequence of the overall size of the Ad genome was also assessed. To adjust the size of the Ad vector genome, we tested a ΔE1/E3AdC68HIVgag37+S vector in which 2500 bp of stuffer sequences had been added. The frequencies of splenic transgene product-specific CD8+ T cells in response to the different vectors, which were assessed in BALB/c mice vaccinated with different doses of vectors, showed that at moderate doses of 10^{10} and 10^{9} vp ΔE1AdC68HIVgag37 and ΔE1/E3AdC68HIVgag37 vectors induced comparable frequencies of gag-specific CD8+ T cells. Addition of stuffer sequences to adjust the genome size had no effect (Figure 3d). The E3 region of adenoviruses encodes polypeptides that serve the virus to evade T-cell-mediated destruction of infected cells by reducing expression of major histocompatibility complex (MHC) class I determinants, by inhibiting TAP-mediated peptide transport into the ER, by blocking fas-mediated apoptosis and the TNF signaling pathways.[25] Circumventing the reduction of expression of

$CD8^+$ T-cell epitopes on MHC class I antigens through deletion of E3 would be expected to increase activation of $CD8^+$ T-cell responses. However, the E1 deletion, which renders Ad vectors replication-defective, also strongly reduces transcription of all other viral genes. Levels of E3 polypeptides encoded for by ΔE1 vectors are hence unlikely to be sufficiently high to influence perceptibly induction of transgene product-specific $CD8^+$ T cells.

Vectors deleted in E1 or E1 and E3 expressing a truncated envelope protein of HIV-1 Clade B, that is, gp140, were also compared. Incorporation of the E3 deletion in addition to E1 for both the AdC68 and AdC7 vectors facilitated incorporation of larger transgenes, such as HIV-1 gp140, but $CD8^+$ T-cell responses to gp140 antigen were similar in both ΔE1 and ΔE1/E3 vectors (not shown).

Effect of transgene length on protein expression and $CD8^+$ T-cell responses

As described above, for some pathogens including HIV-1, vaccines will have to be developed that express multiple antigens. Ad vectors deleted in E1 and E3 accommodate ~7 kb of foreign sequence which suffices to insert gag, pol and nef designed as a fusion gene together with needed regulatory sequences. Alternatively, one could mix vectors expressing individual antigens. The latter would require a dose reduction for each individual vector to accommodate dose-limiting toxicity related to the vaccine carrier. It would also require more cumbersome preclinical toxicity testing prior to phase I trials. Overall, logistically a minimal number of vectors expressing the largest possible amount of foreign sequence would be desirable; nevertheless, the effect of insert length on the ability of simian Ad vectors to induce transgene product-specific $CD8^+$ T-cell has not yet been established. We tested for this by initially comparing simian Ad vectors expressing $SIVmac_{239}$ antigens of different lengths. Ad vectors expressing gag, pol and nef or gag and pol as fusion proteins were compared to Ad vectors expressing gag alone for transgene product expression levels and for induction of gag-specific $CD8^+$ T-cell frequencies in mice. As shown in Figure 4a, levels of protein expression were markedly higher when gag was expressed as an individual antigen. Accordingly, the ΔE1AdC68SIVgag vector induced higher frequencies of transgene product-specific $CD8^+$ T cells compared to ΔE1/E3AdC68SIVgag–pol or ΔE1/E3AdC68SIVgag–pol–nef vectors (Figure 4b).

To extend our T-cell analyses to epitopes other than the one carried by gag we defined $CD8^+$ T-cell epitopes of SIV pol and nef in BALB/c and C57Bl/6 mice. To this end, groups of BALB/c and C57Bl/6 mice were immunized with Ad vectors expressing either gag, pol or nef and splenocytes were tested 14 days later for IFN-γ

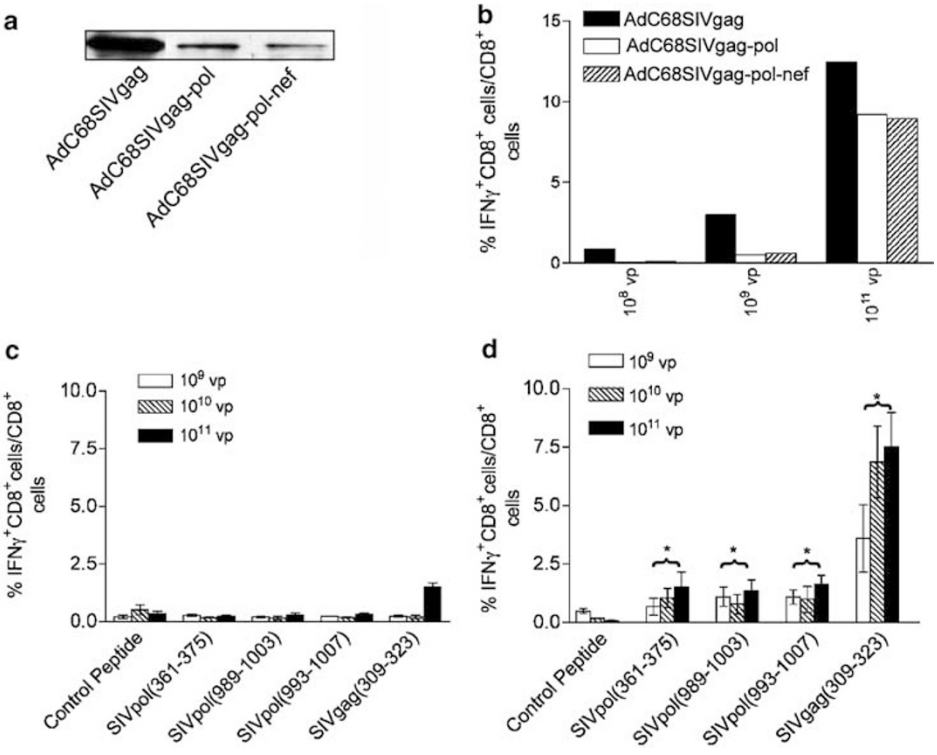

Figure 4 Insert length. (**a**) CHO/CAR cells were infected with 1000 vp/cell of the indicated vectors. After 36 h, cells were harvested, lysed and an equal amount of total protein was used for anti-SIV gag detection. (**b**) C57Bl/6 mice (three per group) were immunized with escalating doses of the indicated vectors. After 10 days, gag-specific $CD8^+$ T-cell frequencies were determined from splenocytes by ICS. (**c**) (C57Bl/6xBALB/c)F1 mice were immunized with ΔE1/E3AdC68SIVgag–pol–nef vector, or (**d**) mixtures of ΔE1AdC68SIVgag, ΔE1/E3AdC68SIVpol or ΔE1AdC68SIVnef vectors. For the fusion protein (**c**), doses were as shown. For the mixture (**d**), each vector was used at 1/3 the dose used for the vector expressing the fusion protein so that both cohorts of mice received the same final dose of vector. Frequencies of transgene product-specific $CD8^+$ T cells were determined by ICS 10 days later from spleens of individual mice using several peptides that carry epitopes for the transgene products. *Indicates *P*-value <0.05 comparing frequencies against a given epitope elicited by either the polypeptide vaccine or the vaccine composed of mixtures of vectors expressing individual antigens.

Table 3 Amino acid sequence of $CD8^+$ peptides

Protein	*Mouse haplotype*	*Sequence*	*Location*
$SIVmac_{239}$gag	$H\text{-}2^d$	QTDAAVKNWMTQTLL	AA 309–323
$SIVmac_{239}$nef	$H\text{-}2^d$	LRARGETYGRLLGEV	AA 21–35
$SIVmac_{239}$nef	$H\text{-}2^d$	GETYGRLLGEVEDGY	AA 25–39
$SIVmac_{239}$pol	$H\text{-}2^d$	WKGSPAIFQYTMRHV	AA 361–375
$SIVmac_{239}$pol	$H\text{-}2^d$	FLMALTDSGPKANII	AA 689–703
$SIVmac_{239}$ pol	$H\text{-}2^d$	NFRVYYREGRDQLWK	AA 989–1003
$SIVmac_{239}$pol	$H\text{-}2^d$	YYREGRDQLWKGPGE	AA 993–1007
$SIVmac_{239}$pol	$H\text{-}2^b$	PAIFQYTMRHVLEPF	AA 365–379
HIV-1gag	$H\text{-}2^d$	AMQMLKETI	AA 197–205
HIV-1env	$H\text{-}2^d$	RGPGRAFVTI	AA 311–320
HIV-1pol	$H\text{-}2^d$	IEELRQHLLRWGFTT	AA 357–371
HIV-1pol	$H\text{-}2^d$	YYRDSRDPLWKGPAK	AA 941–955

production by $CD8^+$ T cells in response to pools of 10–20, overlapping 15mer peptides spanning the sequences of the transgene products. Peptides present in pools that induced a $CD8^+$ T-cell response were retested individually to identify those that carry $CD8^+$ T-cell epitopes. Further mapping of the epitopes to the minimal peptide sequence or identifying their MHC class I restricting molecules in $H\text{-}2^d$ or $H\text{-}2^b$ mice, which is prerequisite for generation of MHC tetramers was not attempted as the identified sequences sufficed to compare frequencies of functionally active $CD8^+$ T cells by intracellular cytokine staining. The identified peptide sequences and their location within the SIV genes as well as other epitope carrying peptides used in this study are shown in Table 3.

To further assess induction of $CD8^+$ T cells by simian Ad vectors expressing gag–pol–nef as a polypeptide or as individual proteins, groups of C57Bl/6 × BALB/c F1 mice were vaccinated with graded doses of the ΔE1/E3AdC68SIVgag–pol–nef vector or mixtures of the three individual AdC68 vectors expressing the same sequences as individual proteins, that is, ΔE1AdC68SIVgag, ΔE1/E3AdC68SIVpol and ΔE1AdC68SIVnef. Vector doses within the mixture were adjusted so that each vector was present at an equal concentration and the final dose of vector (in vp) per mouse equaled that of the polypeptide vaccine. Frequencies of splenic $CD8^+$ T cells to individual epitopes of gag, pol and nef were tested 14 days later. No response could be detected to nef (not shown). As shown in Figure 4c and d, the mixture of vectors expressing individual antigens induced by far higher frequencies of transgene product-specific $CD8^+$ T-cell compared to the polypeptide vaccine even though the final dose of each individual vector in this mixture was only one-third of the dose of the vector expressing the polypeptide.

To test if frequencies of $CD8^+$ T cells to individual epitopes were affected if mice were injected with mixtures of Ad vectors, groups of BALB/c mice were injected with a mixture of four AdC68 vectors expressing antigens of HIV-1. The codon-humanized sequences includes those encoding gp140 of HIV-1 clade B, gag, the N-terminal 650 amino acids of pol of HIV-1 clade B and a construct expressing a modified clade B nef fused at the C-terminus with the remaining 368 amino acids of pol. In this construct, the fusion protein contains a TPA leader sequence fused to the N-terminus of nef. In one group of mice, the vectors designated ΔE1/E3AdC68HIVgp140, ΔE1AdC68HIVgag, ΔE1/E3AdC68HIV5′pol, ΔE1/E3AdC68HIVTPAnef-3′pol were mixed at equal ratios and injected together. In another group, the ΔE1/E3AdC68HIVgp140 was mixed with the ΔE1/E3AdC68HIVTPAnef-3′pol vector and the ΔE1AdC68HIVgag vector was mixed with the ΔE1/E3AdC68HIV5′pol vector and the two mixtures were injected separately into the left or right quadriceps muscles. Additional groups of mice were injected with only one of the vectors. In this experiment, the dose of each individual vector was kept constant and each mouse received 2.5×10^{10} vp of each AdC68 vectors. Responses were tested for by ICS using peptides carrying previously defined epitopes or epitopes that were identified as described above for the SIV antigens (Table 2). As shown in Figure 5b, the effect of mixing vectors expressing different transgene products on frequencies of transgene product-specific $CD8^+$ T cells was largely dependent on the epitope; responses to one of the pol epitopes (AA357–371) were not affected by using mixtures of vector. In contrast, $CD8^+$ T-cell frequencies to the other three epitopes, that is, one additional epitope of pol (AA941–955), one epitope of gag (AA197–205) and one epitope of env (311–320), were markedly higher in mice injected with only the corresponding vector. Application of a mixture of four vectors into one leg versus application of 2 × 2 vectors into different sites resulted in comparable frequencies.

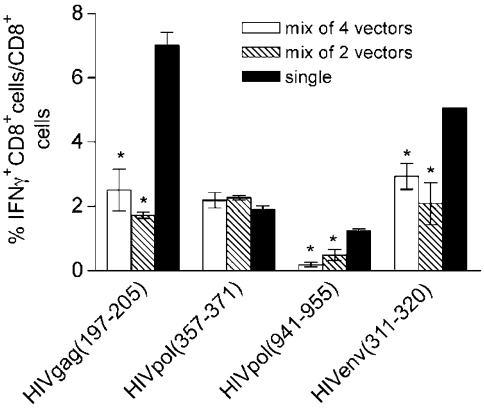

Figure 5 Mixtures of vectors. BALB/c mice (three per group) were immunized with a mixture of ΔE1AdC68HIVgag, ΔE1/E3AdC68HIV5′pol, ΔE1/E3AdC68HIVTPAnef-3′pol and ΔE1/E3AdC68HIVgp140. Each vector was given at 2.5×10^{10} vp per mouse. Vectors were either mixed and injected together (mix of four vectors); ΔE1AdC68HIVgag was mixed with ΔE1/E3AdC68HIV5′pol and ΔE1/E3AdC68HIVTPAnef-3′pol was mixed with ΔE1/E3AdC68HIVgp140 and the two mixtures were injected separately into the right and left quadriceps of mice (mixture of 2 × 2 vectors) or mice were injected with one of the four vectors only (single vectors). Frequencies of transgene product-specific $CD8^+$ T cells were determined by ICS 10 days later from spleens of individual mice. *Represents *P*-value <0.05 comparing frequencies against a given epitope comparing single vectors with vector mixtures.

Discussion

Results of experiments presented in this paper were designed to test the performance of simian replication-

defective Ad vectors as gene transfer vectors for viral antigens especially those derived from pathogens such as HIV-1 for which vaccine-induced protection will rely at least in part on $CD8^+$ T cells. Neutralizing antibodies, the correlate of vaccine-induced protection against most viral infections, that broadly cross-neutralize HIV-1 isolates, cannot currently be induced by vaccines.[26,27] It is assumed based on preclinical studies with SIV that HIV-1 specific T cells will not provide sterilizing immunity or allow for complete clearance of HIV-1 but rather lower the set point viral load and thus reduce morbidity and further spread of the virus. As HIV-1 rapidly mutates in infected hosts, the vaccine-induced $CD8^+$ T-cell response has to be of sufficient breadth to prevent outgrowth of escape mutants. A number of different vaccine carriers have been tested preclinically as well as in part clinically for induction of $CD8^+$ T-cell responses to HIV-1.[28–33]

Here we compared simian Ad vectors that varied with regard to deletions and insert lengths to determine which types of constructs are most suitable for further clinical development. ΔE1AdC68 and ΔE1/E3AdC68 vectors showed favorable growth characteristics and yields commonly exceeded those of AdHu5 vectors. The ratios of infectious to noninfectious particles in AdC68 vectors were comparable to those obtained with AdHu5 vectors. In contrast, AdC7 vectors had, in general higher vp-to-PFU ratios. The effect of deletions in E4 was tested. Deletion of E4 could potentially be advantageous; ΔE4Ad vectors produce less late gene products compared to E1 only deleted vectors and thus reduce induction of vector-specific immune responses and provide less targets for T cells directed to cross-reactive antigens of Ad. Such T cells present in humans with immunological memory to related human serotypes of Ad virus could potentially affect the efficacy of simian Ad vectors. Furthermore, deletion of E4 minimizes outgrowth of replication-competent virus in packaging cell lines, although this is not a major concern for simian-origin Ad vectors grown on packaging cell lines containing the E1 of AdHu5 virus. Previous gene therapy trials reported that deletion or functional inactivation of E4 in AdHu5 vectors reduces the inflammatory response to the vector,[34] and decreases AdHu5-mediated upregulation of adhesion molecules in endothelial cells.[35] Such adhesion molecules are crucial for lymphocyte trafficking. Furthermore, E4 has been reported to affect the performance of viral and cellular promoters.[36] E4 polypeptides have multiple additional functions that may influence the performance of Ad vectors as vaccine carriers. Overall, simian Ad vectors deleted in E4 performed poorly; they showed a decrease in transgene product expression and induced lower frequencies of transgene product-specific $CD8^+$ T cells compared to ΔE1/E3 Ad vectors unless used at high doses.

The length of the insert had the most dramatic effect on performance of the simian Ad vectors expressing antigens of SIVmac239. ΔE1/E3AdC68SIVgag–pol–nef and ΔE1/E3AdC68SIVgag–pol vectors express low levels of antigen and induce only marginal frequencies of transgene product-specific CD8+ T-cell responses when compared to the E1-only deleted vector. Thus although the use of an Ad vector vaccine expressing multiple antigens of SIV/HIV-1 has clear logistic advantages, the poor immunogenicity of such constructs precludes their further development as vaccines. Using mixtures of vectors reduces the magnitude of the $CD8^+$ T-cell response to some of the transgene product. This result was expected and is likely to reflect competition of peptides for the MHC class I peptide binding site. The reduction, although reproducible, is comparatively modest compared to the very strong reduction of the response to vectors expressing the HIV/SIV antigens as long polypeptides. This indicates that for an HIV-1 vaccine where multiple antigens must be expressed, mixtures of simian Ad vectors that achieve high levels of antigen expression should be pursued for clinical development. Single vectors carrying long fusion genes produce less antigen which adversely effects their performance as $CD8^+$ T-cell inducers.

In summary, simian Ad vectors are being developed as vaccines for HIV-1. Results presented here show that ΔE1AdC68 and ΔE1/E3AdC68 vectors have favorable growth characteristics. ΔE1 or ΔE1/E3AdC68 vectors expressing individual antigens or fusion polypeptides of moderate length used as mixtures perform well in small animal immunogenicity studies. This warrants their further exploration for eventual clinical testing.

Materials and methods

Cell culture and virus production

CHO/CAR cells (most kindly provided by J Bergelson, Children's Hospital, University of Pennsylvania, Philadelphia, PA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS, 0.1 mM MEM nonessential amino acids, 1 mM Na pyruvate, 2 mM L-glutamine, 10 mM HEPES, Pen/Strep and Gentamicin in a 10% CO_2 37°C incubator. HEK 293 cells were propagated in DMEM supplemented with the same reagents but for HEPES buffer and cultured in a 5% CO_2 incubator. Adenoviral vectors were generated from molecular clones, propagated, purified and quality controlled (PFU-to-vp ratios, RCA content, genome integrity) as described elsewhere.[11,15] Inserts: clade B SIVgag, SIVgag–pol, SIVgag–pol–nef, SIVpol, SIVnef, HIVgp140 and HIVgag were generous gifts from G Nabel at the NIH/VRC, Bethesda, MD, USA. HIVgag37 was a generous gift from G Pavlakis at the NIH/NCI, Fredrick, MD. The HIV5′pol contains amino acids 1–650 of clade B HIV-1 pol and the HIVTPAnef-3′pol contains a TPA leader sequence fused to clade B HIV-1 nef fused to the C-terminal 368 amino acids of HIV-1 pol. Both HIV5′pol and TPAnef-3′pol were synthesized in codon-optimized forms by Geneart.

Mice

Female BALB/c, C57Bl/6, and (BALB/c × C57Bl/6)F1 mice were purchased at 6–8 weeks of age from Charles River, Jackson Laboratories or Ace Laboratories and housed in the Wistar Institute Animal Facility. Animals were treated according to institutional rules for animal welfare.

Protein expression analysis

For immunoblots 1×10^6 CHO/CAR cells were plated in 6 cm wells and infected with adenoviruses in 500 μl serum-free DMEM for 2 h at 37°C in a 10% CO_2 incubator. They were then supplemented with 2 ml

10% DMEM and cultured for an additional 36 h. Ad was added at either 1000 or 100 virus vp/cell as indicated. After the incubation time, the cells were washed 1 × with phosphate-buffered saline (PBS) containing Ca^{2+} and Mg^{2+} and then incubated on ice for 10 min in hypotonic buffer (10 mM TrisHCl pH8, 5 mM KCl, 2 mM $MgCl_2$ and 100 μM phenyl methyl sulfonyl fluoride (PMSF)). Cells were then scraped into iced microfuge tubes and pulled through a 28 G needle 4–5 × , then spun in a microfuge for 5 min at 12 000 r.p.m. Cell lysates were collected and a Bradford assay was performed to measure total protein recovered. Lysates were boiled for 5 min in Laemmli sample buffer and equal amounts of total protein were loaded onto a 12% PAGE gel. Proteins in the gel were transferred onto PVDF membrane and protein was detected by immunoblot. Antibodies used were 183-H12-5C (NIH AIDS Research and Reference Reagent Program) for HIV-1 Gag at 1:2000 and anti-mouse HRP (Roche) at 1:12 500, for SIV Gag pooled serum from SIV_{mac251}-infected monkeys (MRC AIDS Directed Program) was used at a 1:200 dilution and anti-monkey HRP at a 1:1000 dilution. All incubations and washes were in PBS+0.1%Tween 20; for incubation with the 1° antibody 3% BSA was added. Detection was through luminol reagent Western Lightening (Perkin Elmer).

Intracellular cytokine staining

Splenocytes (1×10^6/sample) were cultured for 5 h at 37°C in 96-well round bottom microtiter plate wells in DMEM supplemented with 2% FBS and 10^{-6} M 2-ME. Brefeldin A (GolgiPlug; BD PharMingen, San Diego, CA, USA) was added at 1 μl/ml. In general, peptides were used for $CD8^+$ T-cell stimulation *in vitro* at a concentration of 1–3 μg/ml. Control cells were incubated with an unrelated peptide or without peptide. After washing, cells were incubated for 30 min at 4°C with 25 μl of a 1/100 dilution of a FITC-labeled Ab to mouse CD8 (BD PharMingen). They were washed again and permeabilized in 1 × Cytofix/Cytoperm (BD PharMingen) for 20 min at 4°C, washed two times with Perm/Wash (BD PharMingen), and incubated in the same buffer for 30 min at 4°C with 25 μl of a 1/100 dilution of a PE-labeled Ab to mouse IFN-γ (BD PharMingen). After washing, cells were examined by two-color flow cytometry using an EPICS Elite XL (Beckman Coulter, Miami, FL, USA), and data were analyzed by WinMDi software. Cells incubated without the specific peptide showed $<0.5\%$ PE staining of $CD8^+$ T cells (data not shown). Data for most experiments, each of which is representative for data obtained in 2–4 separately conducted experiments, reflect the mean frequencies derived from lymphocytes of spleens of 3–5 individual mice in standard deviations (s.d.) of the mean.

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