Broad cellular immunity with robust memory responses to simian immunodeficiency virus following serial vaccination with adenovirus 5- and 35-based vectors

Simon M. Barratt-Boyes,^{1,2} Adam C. Soloff,¹ Wentao Gao,³ Edward Nwanegbo,³ Xiangdong Liu,¹ Premeela A. Rajakumar,⁴ Kevin N. Brown,¹ Paul D. Robbins,⁴ Michael Murphey-Corb,^{1,4} Richard D. Day^{1,5} and Andrea Gambotto^{3,4,6}

Correspondence

Simon M. Barratt-Boyes
smbb@pitt.edu

^{1,5}Department of Infectious Diseases and Microbiology¹ and Department of Biostatistics⁵, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, PA 15261, USA

^{2,3,4,6}Department of Immunology², Department of Surgery³, Department of Molecular Genetics and Biochemistry⁴ and Department of Medicine⁶, School of Medicine, University of Pittsburgh, Pittsburgh, PA 15261, USA

Adenovirus serotype 35 (Ad35) is a promising vaccine platform for human immunodeficiency virus (HIV) infection and emerging infectious diseases as it is uncommon in humans worldwide and is distinct from Ad5, the major vaccine serotype for which many individuals have pre-existing immunity. The immunogenicity of a first-generation, replication-competent Ad35-based vaccine was tested in the simian immunodeficiency virus (SIV) rhesus macaque model by evaluating its capacity to boost immunity generated by Ad5-based vectors. A series of four immunizations with replication-defective Ad5 vectors expressing SIVmac239 *gag* induced high-frequency responses mediated by both CD8⁺ and CD4⁺ T cells directed against several epitopes. Ad5-specific neutralizing antibody responses that did not neutralize Ad35 were rapidly induced but waned over time. Subsequent immunization with Ad5-based vectors was minimally effective, whereas immunization with Ad35-based vectors generated a strong increase in the frequency of Gag-specific T cells with specificities that were unchanged. While this boosting response was relatively transient, challenge with the distinct pathogenic isolate SIV/DeltaB670 generated robust and selective recall responses to Gag with similar specificities as induced by vaccination that were elevated for 25 weeks relative to controls. Vaccination had measurable albeit minor effects on virus load. Unexpectedly, regional hypervariability within the Gag sequence of SIV/DeltaB670 was associated with mutation of the conserved CD8⁺ T-cell epitope CM9 without concurrent flanking mutations and in the absence of immune pressure. These findings support the further development of Ad35 as a vaccine vector, and promote vaccine regimens that utilize serial administration of heterologous adenoviruses.

Received 18 August 2005

Accepted 11 October 2005

INTRODUCTION

Recent focus in human immunodeficiency virus (HIV) vaccine development has been on recombinant live viral vectors, including modified vaccinia virus Ankara strain, *Venezuelan equine encephalitis virus*, vesicular stomatitis virus and adenovirus serotype 5 (Ad5). These viruses have shown promise in monkey immunodeficiency virus models (Amara *et al.*, 2001; Barouch *et al.*, 2001; Casimiro *et al.*, 2003; Davis *et al.*, 2000; Patterson *et al.*, 2004; Rose *et al.*, 2001; Shiver *et al.*, 2002). Adenovirus-based vaccines in particular are being tested in a number of other emerging infectious diseases, including Ebola virus infection and severe acute respiratory syndrome (Gao *et al.*, 2003b; Sullivan *et al.*, 2003).

However, a major limitation of Ad5-based vectors is immunogenicity of the vector itself, which substantially limits boosting with the same strain (Juillard *et al.*, 1995; Santra *et al.*, 2005; Yang *et al.*, 1995). A significant proportion of humans worldwide also have a pre-existing immunity to Ad5 (Kostense *et al.*, 2004; Nwanegbo *et al.*, 2004; Vogels *et al.*, 2003), limiting the utility of this vector in the clinical setting.

To counter this problem we and others have developed adenoviral vectors based on uncommon viruses, including human serotypes Ad11, Ad24, Ad34 and Ad35, and chimpanzee serotypes AdC6, AdC7 and AdC68 (Barouch *et al.*, 2004; Farina *et al.*, 2001; Fitzgerald *et al.*, 2003; Gao *et al.*, 2003a; Mei *et al.*, 2003; Pinto *et al.*, 2003; Reyes-Sandoval

et al., 2004; Seshidhar Reddy *et al.*, 2003; Shiver & Emini, 2004; Vogels *et al.*, 2003). A majority of humans do not have detectable neutralizing antibody (Ab) titres to Ad35 (Kostense *et al.*, 2004; Nwanegbo *et al.*, 2004; Seshidhar Reddy *et al.*, 2003; Vogels *et al.*, 2003), making this a promising vector for vaccine delivery and gene therapy. Ad35 is a group B virus that uses CD46 as a cellular receptor as opposed to the coxsackievirus and adenovirus receptor widely used by other adenoviruses including group C Ad5 (Gaggar *et al.*, 2003). Moreover, Ad35 is not cross-neutralized by Ab to Ad5 (Barouch *et al.*, 2004; Vogels *et al.*, 2003), raising the possibility of combining Ad5 and Ad35 in a serial vaccine strategy. Murine studies support the use of Ad35-based vectors in HIV vaccine design (Barouch *et al.*, 2004), and studies in the non-human primate suggest that a combination of heterologous adenoviral vectors is effective at inducing robust cellular and humoral immune responses to HIV Gag (Reyes-Sandoval *et al.*, 2004). The immunogenicity of Ad35-based vectors has not yet been reported in non-human primates.

In the present study, we sought to test the immunogenicity of a prototype Ad35-based vaccine encoding the simian immunodeficiency virus (SIV) mac239 *gag* gene in Indian rhesus macaques. We took advantage of the fact that rhesus macaques do not have a pre-existing immunity to either Ad5 or Ad35 to evaluate a sequential vaccination strategy using recombinant vectors based on both serotypes. To determine the depth of T-cell immunity, we carried out detailed analyses of responses following vaccination and subsequent mucosal challenge with the pathogenic primary virus isolate SIV/DeltaB670. This uncloned virus contains multiple genotypes that are transmitted across the rectal mucosa (Amedee *et al.*, 1995; Trichel *et al.*, 1997), making it a highly relevant model of sexual exposure to HIV.

METHODS

Generation and expression of recombinant adenoviral vectors.

E1/E3-deleted Ad5-p17 and Ad5-p45 expressing two codon-optimized fragments of SIVmac239 *gag* were constructed as described previously (Brown *et al.*, 2003; Gao *et al.*, 2004). Expression of Gag protein in two parts allowed for potential presentation of subdominant epitopes that may otherwise be limited by competition from immunodominant epitopes (Palmowski *et al.*, 2002). E3-deleted replication-competent Ad35-p17 and Ad35-p45 were constructed using the *loxP* recombination method as described previously (Gao *et al.*, 2003a). Briefly, *Sall*-*NotI* fragments of codon-optimized *gag* p17 or *gag* p45 were cloned into the Ad35 shuttle plasmid pAd35E3. Plasmids were linearized with *EcoRV* and cotransfected with *NotI*-digested Ad35 helper virus Ad35E3/EYFP DNA into CRE8 cells. The resultant Ad35-based vectors were produced in HEK293 cells. Protein expression by Ad5- and Ad35-based vectors was confirmed by Western blot analysis of lysates of infected HEK293 cells using the SIV p17-specific and p27-specific monoclonal Abs KK59 and 2F12, respectively (Brown *et al.*, 2003; and data not shown).

Animals. Eleven adult Indian rhesus macaques (*Macaca mulatta*) housed at the University of Pittsburgh Primate Facility for Infectious Disease Research were used in this study in compliance with institutional regulations. Molecular major histocompatibility complex

(MHC) class I typing for the rhesus macaque alleles Mamu-A*01, A*02, A*08, A*11, B*01, B*03, B*04 and B*17 was carried out through a contract with the Wisconsin National Primate Research Center.

Immunization and SIV challenge. Vaccine viruses were thawed and suspended in saline in separate syringes at a concentration of 10^{11} virus particles per 150 μ l. Diluted viruses were kept cold and injected within 1 h of thawing. Viruses expressing p17 or p45 were given at separate sites by intramuscular injection in the lateral thigh or by intradermal injection in the inguinal region in sedated animals, respectively. Vaccinated and control monkeys were inoculated with an undiluted stock of the primary virus isolate SIV/DeltaB670 by atraumatic instillation into the rectum as described previously (Fuller *et al.*, 2002).

ELISPOT assays. Effector T-cell responses to SIV antigens were analysed in previously frozen peripheral blood mononuclear cells (PBMC) by IFN- γ ELISPOT assay as described previously (Brown *et al.*, 2003). Individual 15 mer peptides at >80% purity representing Gag, Pol, Env and Nef sequences of SIVmac239 and overlapping by 11 aa (NIH AIDS Research & Reference Reagent Program) were dissolved in DMSO and used as antigens. Gag peptides were used in pools of eight peptides or 30–32 peptides ($3.1\text{--}3.9\text{ }\mu\text{g ml}^{-1}$), or as individual peptides ($5\text{ }\mu\text{g ml}^{-1}$) as described previously (Brown *et al.*, 2003). Env and Nef peptides were used as single pools of 212 peptides ($0.6\text{ }\mu\text{g ml}^{-1}$) and 64 peptides ($1.6\text{ }\mu\text{g ml}^{-1}$), respectively. Pol peptides were split into two pools of 131 and 132 peptides ($1\text{ }\mu\text{g ml}^{-1}$). Responses that were two times that of the background with a minimum number of spots of 10 per 200 000 cells were scored as positive.

Analysis of neutralizing Ab responses to adenoviral vectors.

Serum neutralizing Abs to Ad5 and Ad35 were measured using E1/E3-deleted Ad5 expressing enhanced green fluorescent protein and E3-deleted Ad35 expressing enhanced yellow fluorescent protein, respectively, as described previously (Nwanegbo *et al.*, 2004). The end-point titre was calculated as the highest serum dilution that inhibited adenovirus infection of A549 cells by $\geq 50\%$.

Virus quantification and sequence analysis.

Quantification of virion-associated RNA in plasma was performed by real-time PCR as described previously (Fuller *et al.*, 2002). For sequence determination, viral RNA was isolated from cell-free plasma of SIV-infected monkeys using viral RNA mini kit (Qiagen). To amplify the *gag* gene, first-strand cDNA synthesis was primed with random hexamers or the *gag*-specific primer BGAGR: 5'-GCGCTGCAGTGGGAGTTGCCCTGGTGTCACT-3' and reverse transcribed using Superscript II reverse transcriptase (Invitrogen). PCR-amplified fragments containing 90% of the *gag* gene were generated by using the primers BGAGF: 5'-GGCGAATTCATGGGCGTGAGAACTCCGTCTTG-3' and BGAGR with the Expanded High Fidelity PCR system (Roche Applied Science), as per manufacturer's instructions, using an annealing temperature of 53 °C. For analysis of individual cloned viral cDNA sequences, amplicon DNA was purified from agarose gels and cloned into the pGEM-TA vector (Promega) prior to transformation into bacteria. Plasmid DNA was sequenced with a 3770 DNA analyser (Applied Biosystems). Sequence data were aligned with SIVmac239 (GenBank accession no. M33262) using CLUSTAL W.

Statistical analyses. To enable comparison of Gag-specific T-cell responses following virus challenge between vaccinated and control animals, data for each animal were first averaged over predetermined intervals and the mean for all animals in a group was calculated. Mean values for the experimental versus the control animals were then compared over the sequential time points using the non-parametric binomial (sign) test (Day *et al.*, 1999), which examines the consistency of binary differences ($\pm$) between the two groups across time (Fisher & van Belle, 1993). The same method was followed for comparison of virus loads over time in vaccinated and control groups.

RESULTS

Vaccination with Ad35-based vectors boosts immunity induced by Ad5-based vectors

Eight monkeys expressing various MHC class I alleles were immunized either by intramuscular ($n=4$) or intradermal ($n=4$) injection with 10^{11} virus particles each of Ad5-p17 and Ad5-p45 using a series of four immunizations. Two monkeys received a subsequent series of boosts with the same vectors, whereas the remaining six animals received immunizations with the Ad35-based vectors (Table 1). While a conventional replication-defective Ad5-based vector was used, we employed a replication-competent Ad35-based system, as E1-complementing cell lines necessary for the generation of E1-deleted vectors were still under development at the time the experiment was initiated.

All monkeys responded to Ad5-based vaccination as determined by ELISPOT assay, with no clear distinction between administration of vaccines by intramuscular or intradermal injection (Fig. 1). The frequency of IFN- γ -producing Gag-specific effector T cells in uncultured PBMC ranged from 1:1000 to 1:500 in a majority of animals after one to two vaccinations, confirming that vaccination with Ad5-based vectors is highly efficient in monkeys (Casimiro *et al.*, 2003; Shiver *et al.*, 2002). As expected, the initial series of vaccinations with Ad5-based vectors resulted in rapid induction of high titre Ad5-specific serum neutralizing Ab that did not cross-neutralize Ad35 (Fig. 1). Repeated boosting with Ad5-based vectors in this first series of four immunizations generally had limited effect. However, over time Ad5-specific neutralizing Ab titres waned in all animals, declining by a mean of 40-fold over the 27.3 ± 2.8 weeks

before the second series of immunizations (Fig. 1). This drop in titre likely facilitated the weak boosting of Gag-specific immune responses in M1501 at week 51 when Ad5-based vectors were used again, although no such boosting was noted in animal M1601 (Fig. 1). In contrast, vaccination with Ad35-based vectors resulted in a substantial boost of immunity to levels above that initially induced by Ad5-based vaccination in the six remaining animals (Fig. 1). Gag-specific effector T-cell frequencies in uncultured PBMC ranged from 1:500 to 1:250 in M7801, M1701 and M2301 after Ad35-based vaccination, demonstrating the potency of vaccination (Fig. 1). Generally, single vaccination with Ad35-based vectors induced minimal or no vector-specific neutralizing Ab responses, and in five of six animals the titre was undetectable at the time of the boost injection 9 weeks later, allowing significant boosting of Gag-specific responses (Fig. 1). Notably, in M2201 and M1701 the Ad35-specific neutralizing Ab titre dropped to undetectable levels by 10 weeks and 3 months after the second immunization with Ad35, respectively (Fig. 1 and data not shown).

Vaccination and boosting induces broad cellular immunity to Gag

We next sought to characterize in detail the specificity of the T-cell response to Gag following Ad5-based vaccination and Ad35-based boosting in a subset of animals. To do this, we generated pools of eight peptides using a matrix design where each peptide is present in two pools. Candidate peptides were identified from the pools and tested individually to confirm reactivity (Brown *et al.*, 2003). Animal M1701 responded to peptides p35/p36 and p68/p69, each containing common 11 aa sequences, indicating that robust immune responses were directed towards two different

Table 1. Animal characteristics and schedule of immunization and challenge

ID	Age (year), sex	Mamu MHC class I	Vaccine route	Ad5-p17 and Ad5-p45* (week)	Ad35-p17 and Ad35-p45† (week)	SIV/DeltaB670 challenge‡ (week)
M1601	6, M	NE§	i.m.	0, 4, 16, 26, 56, 65	–	77
M1501	5, M	A*08	i.d.¶	0, 4, 16, 26, 51, 60	–	72
M7801	3, M	A*08	i.m.	0, 5, 16, 26	56, 65	77
M1701	5, M	NE	i.m.	0, 4, 16, 26	64, 73	84
M2201	6, M	A*01	i.m.	0, 5, 16, 26	63, 72	84
M2301	5, M	A*08	i.d.	0, 4, 16, 26	51, 60	72
M9700	3, M	A*01, B*01	i.d.	0, 4, 16, 26	43, 52	64
M10201	3, M	A*08	i.d.	0, 4, 16, 26	43, 52	64
M3398	8, M	A*02, B*17	–	–	–	0
M15001	5, M	A*01	–	–	–	0
M14301	3, M	A*01, A*02	–	–	–	0

* 10^{11} virus particles each of E1/E3-deleted Ad5-based vector.

† 10^{11} virus particles each of E3-deleted Ad35-based vector.

‡Atraumatic intrarectal inoculation.

§None of the alleles tested was expressed.

||Intramuscular.

¶Intradermal.

regions of Gag. Boosting with Ad35-based vectors at weeks 64 and 73 did not alter the specificity and breadth of response primed by vaccination with Ad5-based vectors (Fig. 2a). Similarly, animal M7801 responded to two discrete regions of Gag following Ad5-based vaccination represented by peptides p14/p15 and p35/p36, with the same responses being boosted following Ad35-based boosts. Animal M2301 responded to eight peptides representing six different regions of Gag through vaccination (Fig. 2a). As expected, animals M2201 and M9700, which expressed the Mamu-A*01 allele,

Fig. 1. Ad35-based vectors boost immunity to SIV Gag induced by Ad5-based vectors. Monkeys were immunized and boosted with replication-defective Ad5-p17 and Ad5-p45 alone (arrows, top panels) or prior to boosting with replication-competent Ad35-p17 and Ad35-p45 (arrowheads, bottom panels). Left, PBMC were incubated with four pools of Gag peptides and IFN- γ -producing cells were quantified 24 h later. Shown are mean $\pm$ SEM of triplicate determinations for all pools combined after subtraction of background. Thresholds for significance are shown by horizontal lines denoting mean background responses over all time points for each animal. Right, Ad5- and Ad35-specific neutralizing Ab titres in serum. SFC, Spot-forming cells.

responded almost exclusively to peptides p45/p46 containing the immunodominant MHC class I-restricted CD8⁺ T-cell epitope CM9 (Gag_{181–189}) (Miller *et al.*, 1991) (Fig. 2a and data not shown). To determine whether the major peptide-specific responses noted in these animals were MHC class I- or class II-restricted, we depleted CD8⁺ or CD4⁺ T cells, respectively, from PBMC prior to ELISPOT analysis (Brown *et al.*, 2003). IFN- γ release by PBMC from M1701 and M2301 to peptides p68 and p69, respectively, was completely abrogated when CD8⁺ but not CD4⁺ T cells were depleted, indicating that these responses were MHC class I-restricted. IFN- γ responses to peptide p35 by PBMC from M7801 were also CD8⁺ T-cell-dependent (Fig. 2b). In contrast, cytokine release by M7801 PBMC to peptide p15 was dependent upon CD4⁺ T cells and was therefore MHC class II-restricted (Fig. 2b). Collectively, these data indicate that adenovirus-based vaccination induces broad CD4⁺ and CD8⁺ T-cell immunity to Gag.

Robust recall responses to Gag following virus challenge as a function of vaccination

The Gag-specific T-cell response following boosting with Ad35-based vectors was marked but transient, with the frequency of antigen-specific T cells declining rapidly following the boost in most animals (Fig. 1). This raised the concern that the vaccine regimen may not have induced sustained memory responses. To test this directly, we challenged vaccinated and three control animals with the pathogenic uncloned isolate SIV/DeltaB670 via atraumatic intrarectal inoculation 11–12 weeks after the final boost (Table 1). This virus is related but distinct from SIVmac239 on which the vaccine was based (Amedee *et al.*, 1995; Trichel *et al.*, 1997). The two animals vaccinated with Ad5 alone had recall responses to Gag following infection, although the response in M1501 was minimal, despite the minor boost to Gag-specific T-cell responses generated by Ad5-based immunization in this animal. M1501 died of anaesthetic complications unrelated to SIV infection at week 15 post-infection (Fig. 3a). The six animals vaccinated with Ad5- and Ad35-based vectors generally had robust recall responses to Gag as a function of infection, although animal-to-animal variation was considerable (Fig. 3a). In contrast, the three control animals had poor Gag-specific responses following mucosal challenge with SIV/DeltaB670 (Fig. 3b). When

Fig. 2. Vaccination induces broad cellular responses to SIV Gag. (a) PBMC were incubated with diluent or individual Gag peptides at various times after immunization as indicated, and IFN- γ -producing cells were quantified 24 h later. Positive responses as defined in Methods are indicated by asterisks. Shown are mean $\pm$ SEM of triplicate determinations. (b) PBMC from animals M1701, M7801 and M2301 before or after Ab-mediated depletion of CD4 $^{+}$ or CD8 $^{+}$ T cells were incubated with individual 15 mer peptides as indicated and IFN- γ -producing cells were quantified 24 h later. Shown are mean $\pm$ SEM of triplicate determinations based on absolute number of cells in the assay with or without depletion. C, Diluent control; SFC, spot-forming cells; wk, weeks; dep, depleted.

the frequency of Gag-specific responses following infection was compared between animals receiving the Ad5- and Ad35-based vaccinations and control animals, the mean response was greater in vaccinated animals for at least 25 weeks post-infection ($P < 0.0175$; Fig. 3c). This recall response was specific to the vaccine antigen, as cellular responses to Env, Pol and Nef were similar between the Ad5/Ad35 vaccine group and the control group post-challenge (Fig. 3d).

Given that the vaccine and challenge strains of virus were distinct, with overall amino acid sequence dissimilarity within matrix and capsid proteins between SIVmac239 and the multiple genotypes of SIV/DeltaB670 inoculum being 8% (data not shown), we next wanted to determine whether the specificity of Gag-specific immunity induced following challenge differed from that induced through vaccination. Virus infection boosted responses to the same panel of peptides in monkeys M1701 and M2301 as seen following vaccination, with the exception that responses to p23 and p47 were not detected following challenge in monkey M2301. Peak responses in both of these monkeys post-infection were directed against the p68/p69 region containing the CD8 $^{+}$ T-cell epitope(s) identified following vaccination (Fig. 4). Animal M7801 had increased CD8 $^{+}$ T-cell responses to peptides p35/p36 following virus challenge but no boost in the CD4 $^{+}$ T-cell response to peptides p14/p15 (Fig. 4),

despite complete sequence identity in this region between vaccine and challenge strains (data not shown). This animal had new responses to p1 and p67–p69 peptides as a result of infection. As expected, animal M2201 had marked increases post-challenge in the T-cell response to peptides p45/p46 containing the immunodominant and conserved Mamu-A*01-restricted CM9 epitope, approaching a frequency of 0.2% in unseparated PBMC (Fig. 4). This is in noticeable contrast to the negligible responses of the two Mamu-A*01-expressing control animals M14301 and M15001 to Gag following challenge (Fig. 3b). Animal M3398, the single control animal for which a robust post-challenge Gag-specific response was detected, had responses directed exclusively to the p68/p69 region (Fig. 4). Collectively, these data indicate that vaccination induced robust memory responses to SIV Gag that were selectively recalled after virus challenge.

Minor effect of vaccination on virus load

To determine the effect of vaccination on virus load, we measured the copies of viral RNA in plasma in infected monkeys by real-time PCR. As expected a range of virus loads was seen in each group, although animals in the Ad5/Ad35 vaccine group tended to have lower virus loads compared with controls, with animals M1701 and M2301 having repeated measurements that were below the level of detection up to 44 weeks post-challenge (Fig. 5a). Overall,

Fig. 3. Vaccination induces durable recall responses to Gag following SIV infection. Vaccinated (a) or control (b) monkeys were challenged with SIV/DeltaB670 by atraumatic intrarectal inoculation. PBMC were incubated with diluent or Gag peptide pools at various times after challenge and IFN- γ -producing cells were quantified 24 h later. Shown are mean $\pm$ SEM of triplicate determinations for all Gag peptides after subtraction of background. Thresholds for significance are shown by horizontal lines denoting mean background responses over all time points for each animal. (c) Mean Gag-specific IFN- γ responses of Ad5/Ad35-vaccinated and control groups at intervals after virus challenge. Responses over time were compared using a binomial test. (d) Responses of Ad5/Ad35-vaccinated and control animals to Env, Pol and Nef peptide pools at intervals after virus challenge. Shown are mean responses of triplicate determinations after subtraction of background for each animal, with the mean for the group represented by a horizontal line. If more than one sample was analysed during the interval indicated, the stronger response is shown. SFC, Spot-forming cells; vac, vaccinated; con, control.

monkeys in the Ad5/Ad35-vaccinated group had lower mean virus loads as compared with control animals at 11 of 12 time intervals, a finding that is statistically significant ($P=0.003$). However, the magnitude of the difference between groups exceeded 1 log only at two time intervals, being 9–12 weeks and 31–35 weeks post-infection, and was not significant over time (Fig. 5b). Control animals had a median time to death from AIDS of 41.0 weeks compared with 49.3 weeks for the Ad5/Ad35-vaccinated animals, a difference that did not reach significance (log rank test $P=0.094$) (Fig. 2c). Animal M2301 remains alive without AIDS at 70 weeks post-infection. These data demonstrate

that vaccination had a measurable, albeit small, effect on virus load.

Novel mechanism of virus escape due to virus variability

Animal M2201 had a rapid increase in plasma virus load beginning at week 15 post-infection and subsequently died of AIDS at week 32 (Fig. 5) despite having a potent T-cell response to the Mamu-A*01-restricted CM9 epitope (Fig. 4), suggesting that T-cell activity directed towards this epitope was no longer effective. To test for virus escape from

Fig. 4. Infection with a distinct SIV isolate generates responses with similar specificity to those elicited by vaccination. PBMC were incubated with diluent or individual Gag peptides at various times after infection with SIV/DeltaB670 as indicated, and IFN- γ -producing cells were quantified 24 h later. Positive responses as defined in Methods are indicated by asterisks. Shown are mean $\pm$ SEM of triplicate determinations. C, Diluent control; SFC, spot-forming cells; wk, weeks.

the CM9 coding sequence and the surrounding region with the SIVmac239 vaccine sequence. All species of the SIV/DeltaB670 inoculum expressed the CM9 epitope, but there were multiple variations in the flanking sequence when compared with SIVmac239 (Fig. 6a). Escape mutations in the CM9 epitope were found in viruses isolated from three of four Mamu-A*01-expressing animals, with 63 and 100 % of virus clones from animal M2201 harbouring escape mutations by weeks 15 and 23 post-infection, respectively, coincident with the increase in virus load (Fig. 6a). The control-vaccinated animal M15001 had epitope escape mutations in 100 % of virus clones present at week 19 despite minimal Gag-specific T-cell responses to virus (Figs 3b and 6a). Notably, different mutations within the CM9-coding sequence were found in viruses isolated at several time points from the non-Mamu-A*01-expressing animal M1701, with 40 and 75 % of clones isolated at weeks 45 and 62, respectively, expressing mutations (Fig. 6b). Peptides spanning this sequence and flanking regions did not elicit IFN- γ responses during acute and chronic stages of infection in this animal, indicating that mutation did not accompany a detectable T-cell response to an epitope overlapping the CM9 region (Fig. 6c). No consistent extra-epitopic mutations temporally associated with CM9 mutations could be identified.

DISCUSSION

Several novel serotypes of adenovirus are being explored as the basis of vaccines for HIV and other emerging infectious diseases (Barouch & Nabel, 2005; Mei *et al.*, 2003; Shiver & Emini, 2004; Vanniasinkam & Ertl, 2005). Ad35 has been the focus of considerable research based on its low seroprevalence in the global human population and its distinction as a group B virus that is not cross-neutralized by Abs to Ad5 (Gao *et al.*, 2003a; Kostense *et al.*, 2004; Nwanegbo *et al.*, 2004; Seshidhar Reddy *et al.*, 2003; Vogels *et al.*, 2003). Murine studies have demonstrated that Ad35-based vaccines are immunogenic in the face of immunity to Ad5 (Barouch *et al.*, 2004), and we now extend those findings to the preclinical non-human primate model. Our study utilized a first-generation, replication-competent Ad35 vector. Preliminary studies showed that Ad35 infection was subclinical in monkeys and that the virus was not readily transmitted, as infectious Ad35 could not be recovered from saliva, serum or urine following intramuscular injection with Ad35-expressing enhanced yellow fluorescence protein (data not shown). However, replicating Ad35

cytotoxic T cells, we sequenced the virus inoculum and viruses isolated from plasma after infection of the four animals expressing Mamu-A*01 (Table 1) and compared

is not ideal for vaccine development, particularly as the virus can cause disease in elderly and immunosuppressed individuals (Sanchez *et al.*, 1997). With the development

Fig. 5. Ad5- and Ad35-based vaccination with SIV Gag produces measurable but small effects on virus load following challenge. (a) Plasma virus load of control animals and animals vaccinated with Ad5-based or Ad5- and Ad35-based vectors. Final measurements are at the time of sacrifice due to AIDS except for animal M1501 which died of unrelated causes at week 15, and animal M2301 which remains alive and free from disease at week 70 post-infection. (b) Mean virus loads of Ad5/Ad35-vaccinated and control groups at predetermined intervals after challenge. Responses over time were compared using a binomial test. (c) Kaplan-Meier survival curves for Ad5/Ad35-vaccinated and control groups.

of E1-deleted Ad35 packaging cell lines we now have the capacity to generate replication-defective Ad35-based vectors that will be used in future studies (Gao *et al.*, 2003a).

Ad35-based vaccination boosted but did not expand the T-cell repertoire primed by Ad5-based vaccination, which included both CD4⁺ and CD8⁺ T-cell responses. Similarly, other studies have demonstrated the induction of broad T-cell responses to virus in monkeys following adenovirus- and attenuated poxvirus-based vaccination (Casimiro *et al.*, 2003; Hel *et al.*, 2002; Reyes-Sandoval *et al.*, 2004; Santra *et al.*, 2005). While the responses following Ad35-based vaccination in our study were greater than those induced by repetitive Ad5-based vaccination, in general the extent of the increase was not as great as anticipated given the heterologous nature of the vectors. Neutralizing Ab responses to Ad35 were not elicited following Ad5-based vaccination; hence there is no evidence that virus was cleared prior to infection of target cells. It is possible that cross-reactive T-cell immunity to Ad35 was induced through priming immunizations with Ad5, resulting in premature elimination of Ad35-infected cells by adenovirus-specific cytotoxic T cells. A direct comparison of Ad5- and Ad35-based vectors as priming vaccines is needed to determine the relative immunogenicity of the two vector systems. It is interesting to note that serotype-specific neutralizing Abs to both Ad5 and Ad35 waned over time and in the case of Ad35 were undetectable in two animals after two immunizations, suggesting that additional immunizations with the same vector may have been efficacious. Similarly, a boosting response was noted by Casimiro *et al.* (2003) when two immunizations of 10¹⁰ virus particles of Ad5-based vectors were administered 24 weeks apart to rhesus macaques, even in Ad5-experienced monkeys. In contrast, findings by others indicate that two administrations of 10¹² virus particles of Ad5-based vaccines produced sustained neutralizing Ab titres for over 130 weeks that substantially limited T-cell responses to transgenes upon subsequent Ad5 administration (Santra *et al.*, 2005). Taken together these findings suggest that the specific vector dose may significantly influence the durability of vector-specific neutralizing Ab titre and the capacity of subsequent injections with the same vector to boost T-cell immunity.

Fig. 6. Mutation in a conserved immunogenic region of Gag associated with virus variability. (a and b) Sequence comparison between SIVmac239 vaccine strain, SIV/DeltaB670 inoculum and viruses isolated from plasma of Mamu-A*01-expressing animals M2201, M9700, M15001 and M14301 (a) and the non-Mamu-A*01-expressing animal M1701 (b) at the indicated times post-challenge. The Mamu-A*01-restricted CM9 epitope is boxed. Numbers above the sequences represent amino acid positions in the Gag protein, and numbers to the right represent clones carrying the specific sequence. (c) IFN- γ responses of PBMC from animal M1701 to peptides p42–p49 spanning Gag_{165–207} or to p68 or diluent (control) at the times indicated post-infection. Positive responses as defined in Methods are indicated by asterisks. Shown are mean $\pm$ SEM of triplicate determinations. SFC, Spot-forming cells; wk, weeks.

A key goal of vaccination is to induce sustained memory responses that produce enhanced immunity following virus infection. While our serial adenovirus-based regimen induced potent T-cell responses, the duration of these responses prior to challenge was limited. Other similar studies have emphasized the durability of the vaccine response induced through Ad5-based vaccination, although as discussed above these workers used 10-fold higher doses of vector, which may have had an impact on T-cell responses (Santra *et al.*, 2005). Despite the rapid decline of Gag-specific T-cell frequencies after the Ad35-based boosts in our study, the anamnestic T-cell response to Gag following infection with SIV was strong and durable, with increased T-cell frequencies being present as a function of vaccination

for 25 weeks. Of note is the prominent response of a number of animals to the region of capsid represented by peptides p67–p70, which has not previously been defined as immunogenic. We were not able to determine the MHC restriction of these responses; however, epitope mapping using purified 9 mer peptides identified at least two distinct epitopes within this region recognized by PBMC from animal M7801 post-challenge (data not shown). Overall, the immune response post-challenge in our cohort of animals had a detectable, albeit minor, effect on virus load, a finding that is not unexpected given the clear role of other viral antigens in vaccine-induced protection from disease, notably Env (Letvin *et al.*, 2004). Future studies will need to focus on using replication-defective Ad35-based vectors expressing a

range of viral antigens in a priming regimen to test the efficacy of this vaccine system rigorously.

An unexpected finding of our study was the identification of a novel mechanism of virus escape from T-cell recognition. The immunodominant Mamu-A*01-restricted CM9 epitope is highly conserved amongst SIV strains and other lentiviruses, including SIV/DeltaB670 as we now show, and virus escape within the epitope is generally uncommon and slow to evolve in infected macaques (Barouch *et al.*, 2002, 2003; Friedrich *et al.*, 2004a; Peyerl *et al.*, 2003, 2004). Virus escape from T-cell recognition in monkeys infected with SIVmac239 or SHIV-89.6P is associated with flanking mutations that are necessary to maintain *in vitro* replicative fitness (Friedrich *et al.*, 2004a; Peyerl *et al.*, 2003, 2004), although mutated viruses are stable and do not revert to wild-type sequence upon subsequent infection (Friedrich *et al.*, 2004b). While only a small number of Mamu-A*01-expressing animals were followed, our findings are notable in that virus escape from this immunodominant epitope occurred relatively early in the course of infection with SIV/DeltaB670 without any consistent temporal association with flanking mutations. Indeed, the three individual mutations present in viruses from these monkeys are common in CM9 escape mutants following SIV infection and each produce a 100-fold reduction in epitope binding affinity for Mamu-A*01 compared with the wild-type epitope (Barouch *et al.*, 2003). Interestingly, the broader sequence of SIV/DeltaB670 flanking CM9 is hypervariable when compared with SIVmac239 with a dissimilarity of 12%. This includes a valine residue at position 161, which in SIVmac239 isolates is frequently mutated from isoleucine at the time of CM9 epitope escape (Friedrich *et al.*, 2004a). It is conceivable that the stable pre-existing sequence differences in SIV/DeltaB670 impart the capacity for CM9 mutation to occur without incurring fitness costs, thus enabling virus escape relatively early during infection. Consistent with this hypothesis is the discovery that the mutation in the CM9 coding sequence was found in an animal that did not express Mamu-A*01 and which lacked detectable cellular responses to the region. Whether this translates into a lack of a survival advantage in Mamu-A*01-expressing monkeys when infected with SIV/DeltaB670, in contrast to infection with SIVmac251 (Muhl *et al.*, 2002; Palmowski *et al.*, 2002), SIVmac239 (Mothe *et al.*, 2003) and SHIV-89.6P (Zhang *et al.*, 2002), remains to be definitively determined.

ACKNOWLEDGEMENTS

The authors thank D. Slovit for technical assistance, A. Trichel, D. McClemens-McBride, S. Casino, D. Meleason and H. Warnock for assistance with animal procedures, D. Rehrauer for molecular typing of rhesus macaque MHC class I alleles, and C. Rinaldo for access to the ELISPOT reader. This work was supported by Public Health Service grants AI43664 (S.M.B.-B.), AI055794 (S.M.B.-B.) and AI52806 (A.G.) from the National Institutes of Health.

REFERENCES

- Amara, R. R., Villinger, F., Altman, J. D. & 19 other authors (2001). Control of a mucosal challenge and prevention of AIDS by a multiprotein DNA/MVA vaccine. *Science* **292**, 69–74.
- Amedee, A. M., Lacour, N., Gierman, J. L., Martin, L. N., Clements, J. E., Bohm, R., Jr, Harrison, R. M. & Murphey-Corb, M. (1995). Genotypic selection of simian immunodeficiency virus in macaque infants infected transplacentally. *J Virol* **69**, 7982–7990.
- Barouch, D. H. & Nabel, G. J. (2005). Adenovirus vector-based vaccines for human immunodeficiency virus type 1. *Hum Gene Ther* **16**, 149–156.
- Barouch, D. H., Santra, S., Kuroda, M. J. & 13 other authors (2001). Reduction of simian-human immunodeficiency virus 89.6P viremia in rhesus monkeys by recombinant modified vaccinia virus Ankara vaccination. *J Virol* **75**, 5151–5158.
- Barouch, D. H., Kunstman, J., Kuroda, M. J. & 11 other authors (2002). Eventual AIDS vaccine failure in a rhesus monkey by viral escape from cytotoxic T lymphocytes. *Nature* **415**, 335–339.
- Barouch, D. H., Kunstman, J., Glowczwskie, J. & 12 other authors (2003). Viral escape from dominant simian immunodeficiency virus epitope-specific cytotoxic T lymphocytes in DNA-vaccinated rhesus monkeys. *J Virol* **77**, 7367–7375.
- Barouch, D. H., Pau, M. G., Custers, J. H. & 15 other authors (2004). Immunogenicity of recombinant adenovirus serotype 35 vaccine in the presence of pre-existing anti-Ad5 immunity. *J Immunol* **172**, 6290–6297.
- Brown, K., Gao, W., Alber, S., Trichel, A., Murphey-Corb, M., Watkins, S. C., Gambotto, A. & Barratt-Boyes, S. M. (2003). Adenovirus-transduced dendritic cells injected into skin or lymph node prime potent simian immunodeficiency virus-specific T cell immunity in monkeys. *J Immunol* **171**, 6875–6882.
- Casimiro, D. R., Chen, L., Fu, T. M. & 36 other authors (2003). Comparative immunogenicity in rhesus monkeys of DNA plasmid, recombinant vaccinia virus, and replication-defective adenovirus vectors expressing a human immunodeficiency virus type 1 *gag* gene. *J Virol* **77**, 6305–6313.
- Davis, N. L., Caley, I. J., Brown, K. W. & 9 other authors (2000). Vaccination of macaques against pathogenic simian immunodeficiency virus with Venezuelan equine encephalitis virus replicon particles. *J Virol* **74**, 371–378.
- Day, R., Ganz, P. A., Costantino, J. P., Cronin, W. M., Wickerham, D. L. & Fisher, B. (1999). Health-related quality of life and tamoxifen in breast cancer prevention: a report from the National Surgical Adjuvant Breast and Bowel Project P-1 Study. *J Clin Oncol* **17**, 2659–2669.
- Farina, S. F., Gao, G. P., Xiang, Z. Q., Rux, J. J., Burnett, R. M., Alvira, M. R., Marsh, J., Ertl, H. C. & Wilson, J. M. (2001). Replication-defective vector based on a chimpanzee adenovirus. *J Virol* **75**, 11603–11613.
- Fisher, E. R. & van Belle, G. (1993). *Biostatistics: a Methodology for the Health Sciences*. New York, NY: Wiley.
- Fitzgerald, J. C., Gao, G. P., Reyes-Sandoval, A., Pavlakis, G. N., Xiang, Z. Q., Wlazlo, A. P., Giles-Davis, W., Wilson, J. M. & Ertl, H. C. (2003). A simian replication-defective adenoviral recombinant vaccine to HIV-1 *gag*. *J Immunol* **170**, 1416–1422.
- Friedrich, T. C., Frye, C. A., Yant, L. J. & 10 other authors (2004a). Extraepitopic compensatory substitutions partially restore fitness to simian immunodeficiency virus variants that escape from an immunodominant cytotoxic-T-lymphocyte response. *J Virol* **78**, 2581–2585.
- Friedrich, T. C., McDermott, A. B., Reynolds, M. R. & 12 other authors (2004b). Consequences of cytotoxic T-lymphocyte escape: common escape mutations in simian immunodeficiency virus are poorly recognized in naive hosts. *J Virol* **78**, 10064–10073.

- Fuller, D. H., Rajakumar, P. A., Wilson, L. A. & 8 other authors (2002). Induction of mucosal protection against primary, heterologous simian immunodeficiency virus by a DNA vaccine. *J Virol* **76**, 3309–3317.
- Gaggar, A., Shayakhmetov, D. M. & Lieber, A. (2003). CD46 is a cellular receptor for group B adenoviruses. *Nat Med* **9**, 1408–1412.
- Gao, W., Robbins, P. D. & Gambotto, A. (2003a). Human adenovirus type 35: nucleotide sequence and vector development. *Gene Ther* **10**, 1941–1949.
- Gao, W., Tamin, A., Soloff, A., D'Aiuto, L., Nwanegbo, E., Robbins, P. D., Bellini, W. J., Barratt-Boyes, S. & Gambotto, A. (2003b). Effects of a SARS-associated coronavirus vaccine in monkeys. *Lancet* **362**, 1895–1896.
- Gao, W., Rzewski, A., Sun, H., Robbins, P. D. & Gambotto, A. (2004). UpGene: application of a web-based DNA codon optimization algorithm. *Biotechnol Prog* **20**, 443–448.
- Hel, Z., Nacsa, J., Tryniszewska, E. & 7 other authors (2002). Containment of simian immunodeficiency virus infection in vaccinated macaques: correlation with the magnitude of virus-specific pre- and postchallenge CD4⁺ and CD8⁺ T cell responses. *J Immunol* **169**, 4778–4787.
- Juillard, V., Villefroy, P., Godfrin, D., Pavirani, A., Venet, A. & Guillet, J. G. (1995). Long-term humoral and cellular immunity induced by a single immunization with replication-defective adenovirus recombinant vector. *Eur J Immunol* **25**, 3467–3473.
- Kostense, S., Koudstaal, W., Sprangers, M. & 8 other authors (2004). Adenovirus types 5 and 35 seroprevalence in AIDS risk groups supports type 35 as a vaccine vector. *AIDS* **18**, 1213–1216.
- Letvin, N. L., Huang, Y., Chakrabarti, B. K. & 15 other authors (2004). Heterologous envelope immunogens contribute to AIDS vaccine protection in rhesus monkeys. *J Virol* **78**, 7490–7497.
- Mei, Y. F., Skog, J., Lindman, K. & Wadell, G. (2003). Comparative analysis of the genome organization of human adenovirus 11, a member of the human adenovirus species B, and the commonly used human adenovirus 5 vector, a member of species C. *J Gen Virol* **84**, 2061–2071.
- Miller, M. D., Yamamoto, H., Hughes, A. L., Watkins, D. I. & Letvin, N. L. (1991). Definition of an epitope and MHC class I molecule recognized by gag-specific cytotoxic T lymphocytes in SIVmac-infected rhesus monkeys. *J Immunol* **147**, 320–329.
- Mothe, B. R., Weinfurter, J., Wang, C., Rehrauer, W., Wilson, N., Allen, T. M., Allison, D. B. & Watkins, D. I. (2003). Expression of the major histocompatibility complex class I molecule Mamu-A*01 is associated with control of simian immunodeficiency virus SIVmac239 replication. *J Virol* **77**, 2736–2740.
- Muhl, T., Krawczak, M., Ten Haaf, P., Hunsmann, G. & Saueremann, U. (2002). MHC class I alleles influence set-point viral load and survival time in simian immunodeficiency virus-infected rhesus monkeys. *J Immunol* **169**, 3438–3446.
- Nwanegbo, E., Vardas, E., Gao, W., Whittle, H., Sun, H., Rowe, D. T., Robbins, P. D. & Gambotto, A. (2004). Prevalence of neutralizing antibodies to adenoviral serotypes 5 and 35 in the adult populations of The Gambia, South Africa, and the United States. *Clin Diagn Lab Immunol* **11**, 351–357.
- Palmowski, M. J., Choi, E. M., Hermans, I. F. & 10 other authors (2002). Competition between CTL narrows the immune response induced by prime-boost vaccination protocols. *J Immunol* **168**, 4391–4398.
- Patterson, L. J., Malkevitch, N., Venzon, D. & 7 other authors (2004). Protection against mucosal simian immunodeficiency virus SIV(mac251) challenge by using replicating adenovirus-SIV multi-gene vaccine priming and subunit boosting. *J Virol* **78**, 2212–2221.
- Peyerl, F. W., Barouch, D. H., Yeh, W. W., Bazick, H. S., Kunstman, J., Kunstman, K. J., Wolinsky, S. M. & Letvin, N. L. (2003). Simian-human immunodeficiency virus escape from cytotoxic T-lymphocyte recognition at a structurally constrained epitope. *J Virol* **77**, 12572–12578.
- Peyerl, F. W., Bazick, H. S., Newberg, M. H., Barouch, D. H., Sodroski, J. & Letvin, N. L. (2004). Fitness costs limit viral escape from cytotoxic T lymphocytes at a structurally constrained epitope. *J Virol* **78**, 13901–13910.
- Pinto, A. R., Fitzgerald, J. C., Giles-Davis, W., Gao, G. P., Wilson, J. M. & Ertl, H. C. (2003). Induction of CD8⁺ T cells to an HIV-1 antigen through a prime boost regimen with heterologous E1-deleted adenoviral vaccine carriers. *J Immunol* **171**, 6774–6779.
- Reyes-Sandoval, A., Fitzgerald, J. C., Grant, R., Roy, S., Xiang, Z. Q., Li, Y., Gao, G. P., Wilson, J. M. & Ertl, H. C. (2004). Human immunodeficiency virus type 1-specific immune responses in primates upon sequential immunization with adenoviral vaccine carriers of human and simian serotypes. *J Virol* **78**, 7392–7399.
- Rose, N. F., Marx, P. A., Luckay, A. & 7 other authors (2001). An effective AIDS vaccine based on live attenuated vesicular stomatitis virus recombinants. *Cell* **106**, 539–549.
- Sanchez, M. P., Erdman, D. D., Torok, T. J., Freeman, C. J. & Matyas, B. T. (1997). Outbreak of adenovirus 35 pneumonia among adult residents and staff of a chronic care psychiatric facility. *J Infect Dis* **176**, 760–763.
- Santra, S., Seaman, M. S., Xu, L. & 13 other authors (2005). Replication-defective adenovirus serotype 5 vectors elicit durable cellular and humoral immune responses in nonhuman primates. *J Virol* **79**, 6516–6522.
- Seshidhar Reddy, P., Ganesh, S., Limbach, M. P., Brann, T., Pinkstaff, A., Kaloss, M., Kaleko, M. & Connelly, S. (2003). Development of adenovirus serotype 35 as a gene transfer vector. *Virology* **311**, 384–393.
- Shiver, J. W. & Emini, E. A. (2004). Recent advances in the development of HIV-1 vaccines using replication-incompetent adenovirus vectors. *Annu Rev Med* **55**, 355–372.
- Shiver, J. W., Fu, T. M., Chen, L. & 49 other authors (2002). Replication-incompetent adenoviral vaccine vector elicits effective anti-immunodeficiency-virus immunity. *Nature* **415**, 331–335.
- Sullivan, N. J., Geisbert, T. W., Geisbert, J. B., Xu, L., Yang, Z. Y., Roederer, M., Koup, R. A., Jahrling, P. B. & Nabel, G. J. (2003). Accelerated vaccination for Ebola virus haemorrhagic fever in non-human primates. *Nature* **424**, 681–684.
- Trichel, A. M., Roberts, E. D., Wilson, L. A., Martin, L. N., Ruprecht, R. M. & Murphey-Corb, M. (1997). SIV/DeltaB670 transmission across oral, colonic, and vaginal mucosae in the macaque. *J Med Primatol* **26**, 3–10.
- Vanniasinkam, T. & Ertl, H. C. (2005). Adenoviral gene delivery for HIV-1 vaccination. *Curr Gene Ther* **5**, 203–212.
- Vogels, R., Zuijdgheest, D., van Rijnsoever, R. & 20 other authors (2003). Replication-deficient human adenovirus type 35 vectors for gene transfer and vaccination: efficient human cell infection and bypass of preexisting adenovirus immunity. *J Virol* **77**, 8263–8271.
- Yang, Y., Li, Q., Ertl, H. C. & Wilson, J. M. (1995). Cellular and humoral immune responses to viral antigens create barriers to lung-directed gene therapy with recombinant adenoviruses. *J Virol* **69**, 2004–2015.
- Zhang, Z. Q., Fu, T. M., Casimiro, D. R. & 14 other authors (2002). Mamu-A*01 allele-mediated attenuation of disease progression in simian-human immunodeficiency virus infection. *J Virol* **76**, 12845–12854.