JOURNAL OF VIROLOGY, Dec. 2006, p. 12009–12016 Vol. 80, No. 24
0022-538X/06/$08.00+0 doi:10.1128/JVI.01749-06

Immunogenicity of Heterologous Recombinant Adenovirus Prime-Boost Vaccine Regimens Is Enhanced by Circumventing Vector Cross-Reactivity▽

Anna R. Thorner,[1] Angelique A. C. Lemckert,[2] Jaap Goudsmit,[2] Diana M. Lynch,[1] Bonnie A. Ewald,[1] Matthew Denholtz,[1] Menzo J. E. Havenga,[2] and Dan H. Barouch[1]*

Division of Viral Pathogenesis, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts 02215,[1] and Crucell Holland BV, 2301 CA, Leiden, The Netherlands[2]

Received 12 August 2006/Accepted 30 September 2006

The high prevalence of preexisting immunity to adenovirus serotype 5 (Ad5) in human populations has led to the development of recombinant adenovirus (rAd) vectors derived from rare Ad serotypes as vaccine candidates for human immunodeficiency virus type 1 and other pathogens. Vaccine vectors have been constructed from Ad subgroup B, including rAd11 and rAd35, as well as from Ad subgroup D, including rAd49. However, the optimal combination of vectors for heterologous rAd prime-boost vaccine regimens and the extent of cross-reactive vector-specific neutralizing antibodies (NAbs) remain poorly defined. We have shown previously that the closely related vectors rAd11 and rAd35 elicited low levels of cross-reactive NAbs. Here we show that these cross-reactive NAbs correlated with substantial sequence homology in the hexon hypervariable regions (HVRs) and suppressed the immunogenicity of heterologous rAd prime-boost regimens. In contrast, vectors with lower hexon HVR homology, such as rAd35 and rAd49, did not elicit detectable cross-reactive vector-specific NAbs. Consistent with these findings, rAd35-rAd49 vaccine regimens proved more immunogenic than both rAd35-rAd5 and rAd35-rAd11 regimens in mice with anti-Ad5 immunity. These data suggest that optimal heterologous rAd prime-boost regimens should include two vectors that are both rare in human populations to circumvent preexisting antivector immunity as well as sufficiently immunologically distinct to avoid cross-reactive antivector immunity.

Recombinant adenovirus serotype 5 (rAd5) vector-based vaccines have been shown to elicit robust antigen-specific immune responses in preclinical studies (17, 19, 20) and are currently being evaluated in large-scale clinical trials for human immunodeficiency virus type 1 and other pathogens. A potential limitation of rAd5 vectors, however, is that a high percentage of humans have preexisting immunity to Ad5, particularly in the developing world (10, 16, 23, 24). Preexisting anti-Ad5 immunity has been shown to suppress the immunogenicity of rAd5 vaccines in both preclinical studies and clinical trials (2–4, 7, 11, 15, 16, 22, 25). To overcome this problem, several groups have developed novel rAd vaccine vectors derived from rare human Ad serotypes (9, 16, 24) as well as from nonhuman Ad serotypes (6, 7) that evade anti-Ad5 immunity. We have also recently shown that a chimeric rAd5 vector containing the hexon hypervariable regions (HVRs) of Ad48 effectively circumvented anti-Ad5 immunity (14). All of these rAd vectors, however, generate potent antivector immunity that diminishes the utility of homologous vector readministration.

Heterologous rAd prime-boost regimens that include two different rAd vectors can be utilized to enhance antigen-specific responses, although the optimal vectors to include in such regimens remain poorly defined. The 51 known human Ad serotypes are divided into six subgroups, A to F. We previously evaluated the immunogenicity of two vectors derived from Ad subgroup B, rAd11 and rAd35, but their utility in heterologous prime-boost regimens proved limited, presumably as a result of low levels of cross-reactive vector-specific neutralizing antibodies (NAbs) (11). We hypothesized that genetically more divergent rAd vectors that avoid cross-reactive vector-specific NAbs would prove more immunogenic in heterologous rAd prime-boost regimens. However, the immunogenicity of regimens involving two rare serotype rAd vectors that avoid cross-reactive vector-specific NAbs has not been explored previously.

In this study, we evaluate the degree of cross-reactive vector-specific NAbs among four rAd vectors: rAd5 from subgroup C, rAd11 and rAd35 from subgroup B, and rAd49 from subgroup D. We investigate the extent of sequence homology among capsid components of these rAd vectors and define optimal heterologous rAd prime-boost regimens in mice both with and without anti-Ad5 immunity. These studies demonstrate that optimal prime-boost vaccine regimens should include two rAd vectors that evade both preexisting anti-Ad5 immunity and cross-reactive antivector immunity.

MATERIALS AND METHODS

Vector construction, production, and purification. E1/E3-deleted, replication-incompetent rAd5, rAd11, rAd35, and rAd49 vectors expressing the same SIVmac239 Gag gene (where SIV is simian immunodeficiency virus) were generated in E1-complementing PER.C6 cells and purified using CsCl gradients as previously described (8, 9, 24). Viral particles were quantitated by high-performance liquid chromatography.

Animals and immunizations. Six- to eight-week-old C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA). Mice were injected

* Corresponding author. Mailing address: Research East Room 213, Division of Viral Pathogenesis, Beth Israel Deaconess Medical Center, 330 Brookline Avenue, Boston, MA 02215. Phone: (617) 667-4434. Fax: (617) 667-8210. E-mail: dbarouch@bidmc.harvard.edu.

▽ Published ahead of print on 11 October 2006.

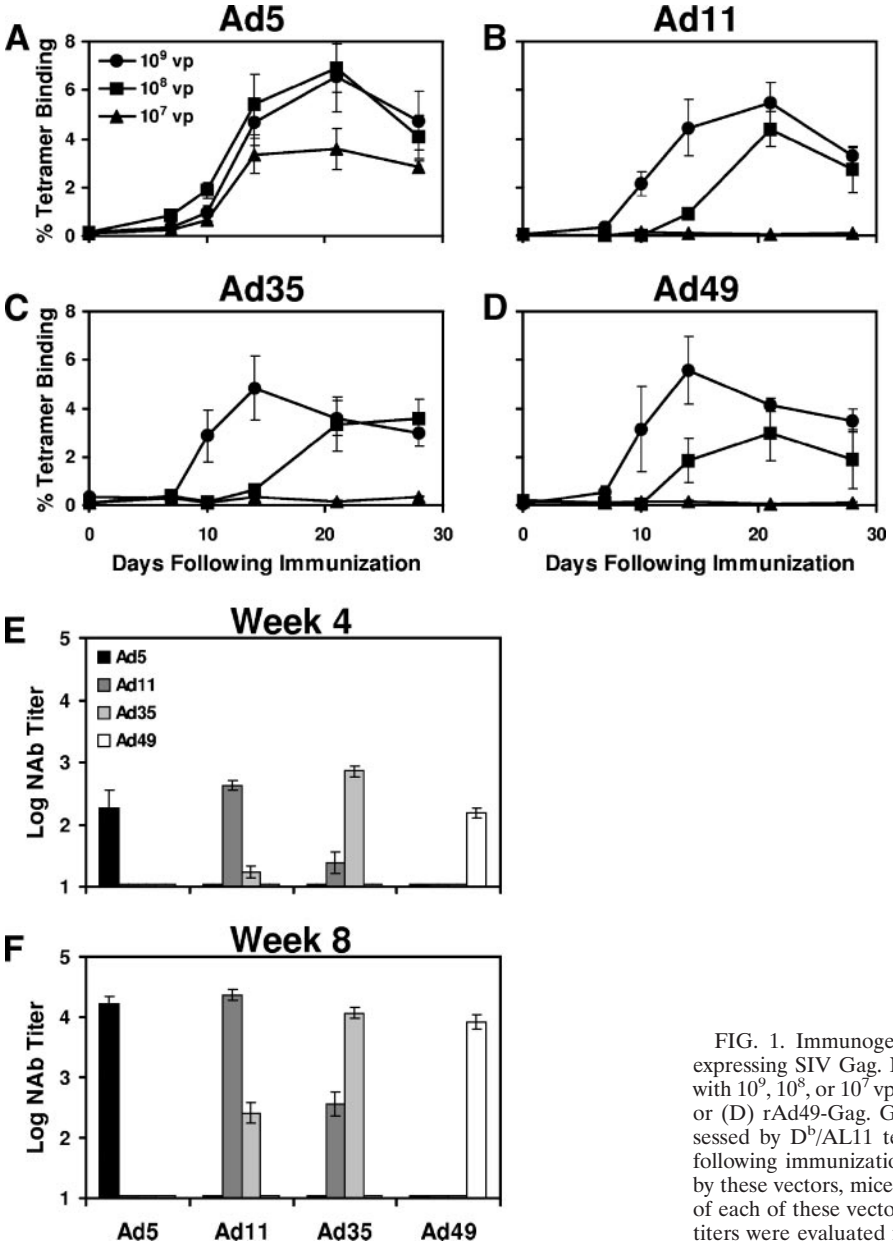

FIG. 1. Immunogenicity of rAd5, rAd11, rAd35, and rAd49 vectors expressing SIV Gag. Naïve mice ($n = 4$/group) were immunized i.m. with 10^9, 10^8, or 10^7 vp (A) rAd5-Gag, (B) rAd11-Gag, (C) rAd35-Gag, or (D) rAd49-Gag. Gag-specific cellular immune responses were assessed by D^b/AL11 tetramer binding assays at multiple time points following immunization. To evaluate vector-specific NAbs generated by these vectors, mice were primed at week 0 and week 4 with 10^{10} vp of each of these vectors. Ad5-, Ad11-, Ad35-, and Ad49-specific NAb titers were evaluated for these mice at (E) week 4 and (F) week 8.

intramuscularly (i.m.) with various doses of replication-incompetent rAd5-Gag, rAd11-Gag, rAd35-Gag, or rAd49-Gag vector in 100 μl sterile phosphate-buffered saline (PBS) in both quadriceps muscles. To induce active antivector immunity, mice were preimmunized once or twice separated by a 4-week interval i.m. with 10^{10} viral particles (vp) rAd5, rAd35, rAd49, rAd35k5 (a chimeric rAd35 vector containing the Ad5 fiber knob), or rAd35k49 (a chimeric rAd35 vector containing the Ad49 fiber knob) expressing either no transgene or luciferase in 100 μl sterile PBS.

Tetramer binding assays. Tetrameric H-2D^b complexes folded around the immunodominant SIV Gag AL11 epitope (AAVKNWMTQTL) (3) were prepared and utilized to stain peptide-specific CD8$^+$ T lymphocytes from C57BL/6 mice as described previously (1, 2, 21). Mouse blood was collected in RPMI 1640 containing 40 U/ml heparin. Following lysis of red blood cells, 0.1 μg of phycoerythrin-labeled D^b/AL11 tetramer in conjunction with allophycocyanin-labeled anti-CD8α monoclonal antibody (Ly-2; Caltag, San Francisco, CA) was utilized to stain AL11-specific CD8$^+$ T lymphocytes. The cells were washed in PBS containing 2% fetal bovine serum (FBS) and fixed in 0.5 ml PBS containing 1.5% paraformaldehyde. Samples were analyzed by two-color flow cytometry with a FACSCalibur instrument (BD Pharmingen, San Diego, CA). Gated CD8$^+$ T lymphocytes were examined for staining with the D^b/AL11 tetramer. CD8$^+$ T lymphocytes from naïve mice were utilized as negative controls and exhibited <0.1% tetramer staining.

ELISPOT assays. Gag-specific cellular immune responses in vaccinated mice were assessed by gamma interferon (IFN-γ) enzyme-linked immunospot (ELIS POT) assays as described previously (3, 21). Overlapping 15-amino-acid peptides spanning the SIVmac239 Gag protein were obtained from the NIH AIDS Research and Reference Reagent Program. Ninety-six-well multiscreen plates (Millipore, Bedford, MA) were coated overnight with 100 μl/well of 10 μg/ml anti-mouse IFN-γ (BD Pharmingen, San Diego, CA) in endotoxin-free Dulbecco's PBS (D-PBS). The plates were then washed three times with D-PBS containing 0.25% Tween 20 (D-PBS–Tween), blocked for 2 h with D-PBS containing 5% FBS at 37°C, washed three times with D-PBS–Tween, rinsed with RPMI 1640 containing 10% FBS to remove the Tween 20, and incubated with 2 μg/ml each peptide and 5×10^5 murine splenocytes in triplicate in 100-μl reaction volumes. Following an 18-h incubation at 37°C, the plates were washed nine times with D-PBS–Tween and once with distilled water. The plates were then incubated

with 2 μg/ml biotinylated anti-mouse IFN-γ (BD Pharmingen, San Diego, CA) for 2 h at room temperature, washed six times with Coulter Wash (Coulter Corporation, Miami, FL), and incubated for 2 h with a 1:500 dilution of streptavidin-alkaline phosphatase (Southern Biotechnology Associates, Birmingham, AL). Following five washes with Coulter Wash and one with PBS, the plates were developed with nitroblue tetrazolium–5-bromo-4-chloro-3-indolylphosphate chromogen (Pierce, Rockford, IL), the reaction was stopped by washing the plates with tap water, and the plates were air dried and read using an ELISPOT reader (Cellular Technology Ltd., Cleveland, OH). The numbers of spot-forming cells per 10^6 cells were calculated. Medium backgrounds were consistently <15 spot-forming cells per 10^6 cells.

Virus neutralization assay. Ad5-, Ad11-, Ad35-, and Ad49-specific NAb titers were assessed by luciferase-based virus neutralization assays as described previously (18). A549 human lung carcinoma cells were plated at a density of 1×10^4 cells per well in 96-well plates and infected with E1/E3-deleted, replication-incompetent rAd5-luciferase, rAd11-luciferase, rAd35-luciferase, or rAd49-luciferase reporter constructs at a multiplicity of infection of 500 with twofold serial dilutions of serum in 200-μl reaction volumes. Following a 24-h incubation, luciferase activity in the cells was measured using a Steady-Glo luciferase reagent system (Promega, Madison, WI) with a Victor 1420 multilabel counter (Perkin Elmer, Wellesley, MA). Neutralization titers were defined as the maximum serum dilution that neutralized 90% of luciferase activity.

Statistical analyses. Statistical analyses were performed with GraphPad Prism, version 4.01 (GraphPad Software, Inc., 2004). Immune responses among groups of mice are presented as means with standard errors. Comparisons of mean immune responses were performed by analyses of variance with Bonferroni adjustments to account for multiple comparisons. In all cases, *P* values of less than 0.05 were considered significant.

RESULTS

Immunogenicity of rAd5, rAd11, rAd35, and rAd49 vectors expressing SIV Gag. We initiated studies to compare the immunogenicities of the rare-serotype vectors rAd11 (9), rAd35 (24), and rAd49 (11a). Groups of naïve C57BL/6 mice (n = 4/group) were immunized once i.m. with 10^9, 10^8, or 10^7 vp of E1/E3-deleted, replication-incompetent rAd5, rAd11, rAd35, or rAd49 vectors expressing SIVmac239 Gag. Vaccine-elicited cellular immune responses were assessed by D^b/AL11 tetramer binding assays (1, 2). As shown in Fig. 1A to D, rAd5-Gag elicited potent $CD8^+$ T-lymphocyte responses at all doses tested, whereas rAd11-Gag, rAd35-Gag, and rAd49-Gag induced responses at 10^9 vp and 10^8 vp but not at 10^7 vp. These data demonstrate that these rare-serotype rAd-Gag vectors were less immunogenic than rAd5-Gag but were comparably immunogenic to each other.

We next evaluated NAb titers elicited by these vectors using luciferase-based virus neutralization assays (18). Mice were immunized at week 0 and week 4 with 10^{10} vp of each of these vectors, and vector-specific NAb titers were assessed at week 4 and week 8. As shown in Fig. 1E and F, rAd11 and rAd35 from subgroup B induced cross-reactive vector-specific NAbs that were particularly evident following the homologous boost immunization. These cross-reactive NAb titers were approximately 1.5 to 2.0 log lower than the NAb titers to the homologous vector. In contrast, we detected no cross-reactive NAbs between rAd49 from subgroup D and the other vectors, including rAd5 from subgroup C and both rAd11 and rAd35 from subgroup B. These data suggest that optimal heterologous rAd prime-boost regimens should include two rare-serotype rAd vectors that are sufficiently immunologically distinct, such as one rAd vector from subgroup B and one rAd vector from subgroup D, to avoid cross-reactive vector-specific NAbs.

To explore in greater detail the molecular basis of these cross-reactive vector-specific NAbs, we compared amino acid sequence homologies in the hexon, penton, and fiber capsid proteins of these vectors. The hexon protein consists of a double barrel core with seven short HVRs extending from its solvent-exposed surface (5). As shown in Table 1, we observed substantial sequence homology in the hexon HVRs between rAd11 and rAd35 (66%) but markedly lower hexon HVR homologies among the other pairs of rAd vectors that did not elicit detectable cross-reactive NAbs (15 to 27%). These data suggest that hexon HVR sequence homology correlates with vector-specific cross-reactive NAbs. This model is also consistent with our previous observations that the HVRs of Ad5 represent primary targets of Ad5-specific NAbs (14, 23). The fiber knobs of these rAd vectors similarly showed higher sequence homology between rAd11 and rAd35 (49%) than among the other pairs of rAd vectors (28 to 48%), although these differences were less striking than for the hexon HVRs. The hexon cores and the pentons of these rAd vectors demonstrated high sequence homology among all of the vectors studied (86 to 98% and 69 to 99%, respectively), suggesting that these relatively conserved regions are not likely the primary determinants of the cross-reactive vector-specific NAbs between rAd11 and rAd35.

TABLE 1. Homology of hexon HVR, hexon core, penton, and fiber knob regions of rAd vectors

rAd vector and region	% Amino acid homology			
	Ad5	Ad11	Ad35	Ad49
Hexon HVR				
Ad5		17	15	21
Ad11			66	27
Ad35				26
Hexon core				
Ad5		86	86	86
Ad11			98	91
Ad35				90
Penton				
Ad5		70	69	71
Ad11			99	76
Ad35				76
Fiber knob				
Ad5		28	29	48
Ad11			49	28
Ad35				29

Immunogenicity of heterologous rAd prime-boost regimens in naïve mice. We next explored the immunogenicity of various heterologous rAd prime-boost regimens in naïve mice. We previously observed that rAd35/rAd11 regimens were less immunogenic than rAd35/rAd5 regimens, but it was not clear from these studies whether this finding reflected the higher intrinsic immunogenicity of rAd5 vectors or the lack of cross-reactive vector-specific NAbs between rAd35 and rAd5 (11). Since rAd11, rAd35, and rAd49 exhibited comparable immunologic potencies (Fig. 1A to D), we primed naïve C57BL/6 mice (n = 4/group) at week 0 with 10^9 vp rAd35-Gag or rAd49-Gag and boosted these mice at week 4 with 10^9 vp rAd5-Gag, rAd11-Gag, rAd35-Gag, or rAd49-Gag. Gag-specific cellular immune responses were assessed by D^b/AL11 tetramer binding assays at multiple time points following im-

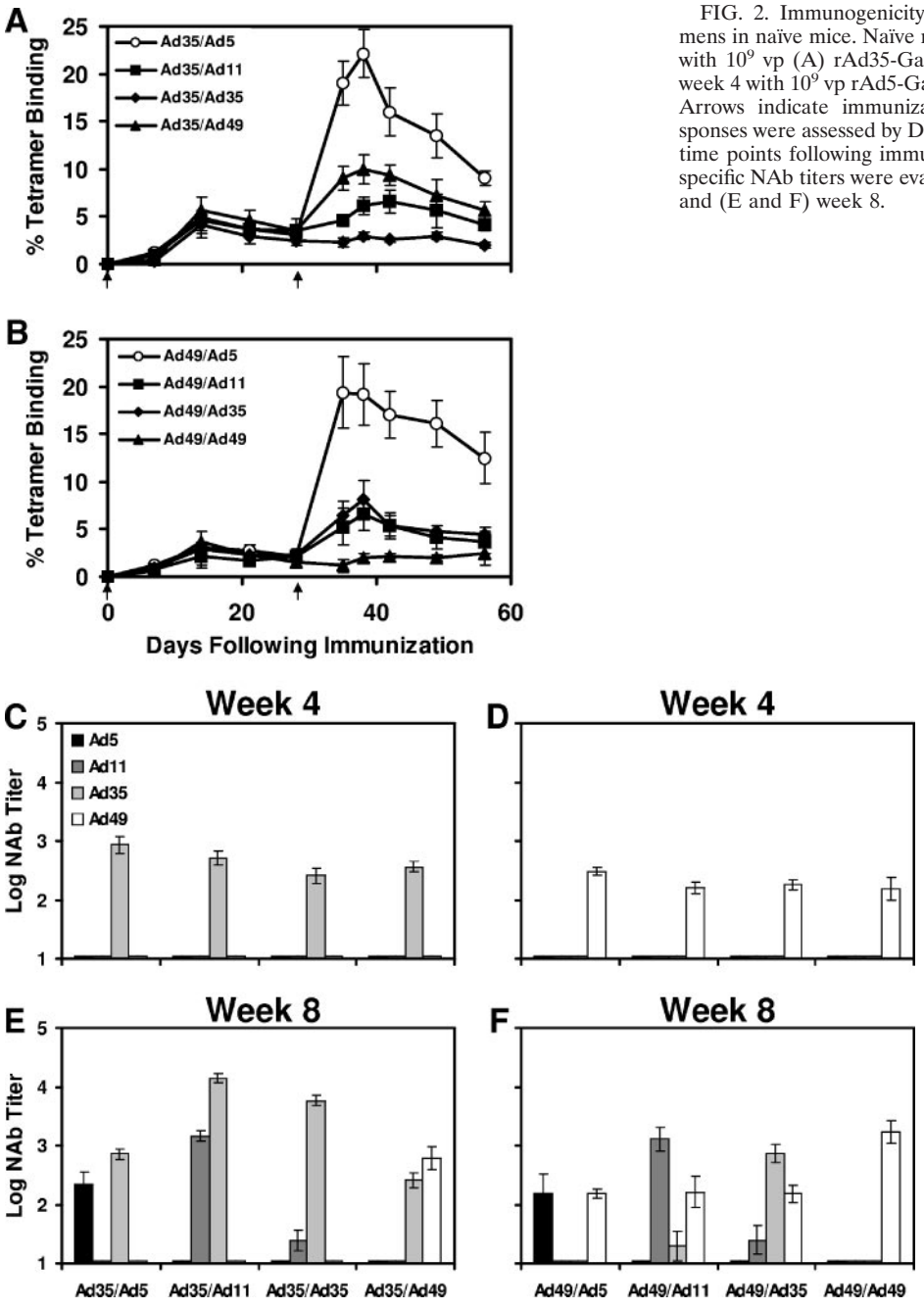

FIG. 2. Immunogenicity of heterologous rAd prime-boost regimens in naïve mice. Naïve mice (n = 4/group) were primed at week 0 with 10^9 vp (A) rAd35-Gag or (B) rAd49-Gag and then boosted at week 4 with 10^9 vp rAd5-Gag, rAd11-Gag, rAd35-Gag, or rAd49-Gag. Arrows indicate immunizations. Gag-specific cellular immune responses were assessed by D^b/AL11 tetramer binding assays at multiple time points following immunization. Ad5-, Ad11-, Ad35-, and Ad49-specific NAb titers were evaluated for these mice at (C and D) week 4 and (E and F) week 8.

munization. As shown in Fig. 2A, mice primed with rAd35-Gag were boosted by rAd5-Gag to peak tetramer-positive $CD8^+$ T-lymphocyte responses of 22.1%, by rAd49-Gag to peak responses of 10.0%, by rAd11-Gag to peak responses of 6.1%, and by rAd35-Gag to 3.0%. These data confirm that heterologous rAd prime-boost regimens are more immunogenic than homologous regimens. Moreover, the rAd35/rAd49 regimen proved more immunogenic than the rAd35/rAd11 regimen ($P < 0.05$, comparing responses on day 38 with analyses of variance and Bonferroni adjustments), presumably due to the lack of detectable cross-reactive NAbs between rAd35 and rAd49 (Fig. 1). The rAd35/rAd5 regimen proved the most immunogenic as a result of the higher intrinsic potency of the rAd5 vector as well as the lack of cross-reactive NAbs between rAd35 and rAd5 ($P < 0.001$). These data suggest that both intrinsic vector potency and immunologic vector cross-reactivity are important for determining the immunogenicity of heterologous rAd prime-boost regimens in naïve mice. Similarly, as shown in Fig. 2B, mice primed with rAd49-Gag were boosted optimally with rAd5-Gag, less well with rAd11-Gag and rAd35-Gag, and poorly with a second immunization of rAd49-Gag.

We evaluated vector-specific NAbs in these mice after both the prime and the boost immunizations. As shown in Fig. 2C and D, mice primed with rAd35 or rAd49 exhibited NAbs

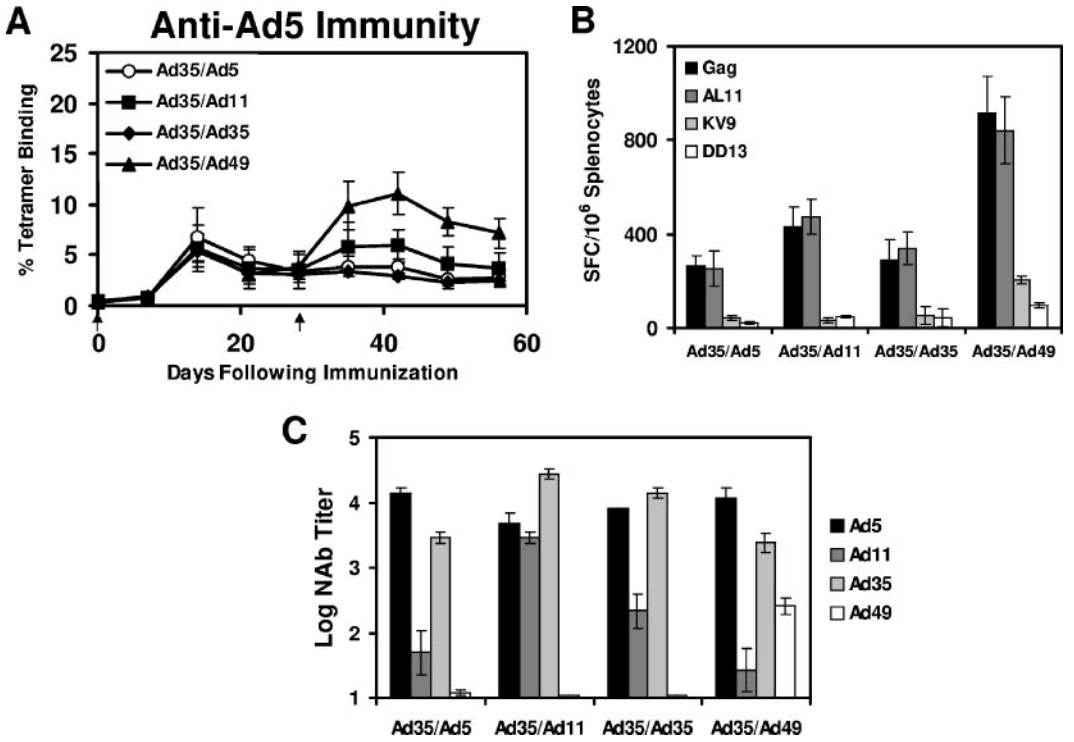

FIG. 3. Immunogenicity of heterologous rAd prime-boost regimens in mice with anti-Ad5 immunity. Mice with anti-Ad5 immunity (n = 4/group) were primed at week 0 with 10^9 vp rAd35-Gag and then boosted at week 4 with 10^9 vp rAd5-Gag, rAd11-Gag, rAd35-Gag, or rAd49-Gag. Arrows indicate immunizations. Gag-specific cellular immune responses were assessed by (A) D^b/AL11 tetramer binding assays at multiple time points following immunization and (B) IFN-γ ELISPOT assays in response to a Gag peptide pool as well as the $CD8^+$ T-lymphocyte epitopes AL11 and KV9 and the $CD4^+$ T-lymphocyte epitope DD13 at week 8. SFC, spot-forming cells. (C) Ad5-, Ad11-, Ad35-, and Ad49-specific NAb titers were evaluated for these mice at week 8.

against the homologous vector at week 4. At week 8 following the boost immunization, mice developed NAbs specific for both the priming vector and the boosting vector. For example, as shown in Fig. 2E, mice primed with rAd35 and boosted with rAd5 developed both Ad35- and Ad5-specific NAbs, whereas mice primed with rAd35 and boosted with rAd49 developed both Ad35- and Ad49-specific NAbs. Interestingly, mice primed with rAd35 and boosted with rAd11 developed both Ad11-specific NAbs and anamnestic Ad35-specific NAbs following the boost immunization. As expected, mice primed and boosted with rAd35 developed high-titer Ad35-specific NAbs as well as low-titer cross-reactive Ad11-specific NAbs. These data demonstrate that highly related rAd vectors elicit not only primarily detectable cross-reactive NAbs but also cross-reactive recall responses following heterologous boost immunizations. Similarly, as shown in Fig. 2F, mice primed with rAd49 and boosted with rAd5, rAd11, rAd35, or rAd49 developed the expected patterns of vector-specific NAb titers following the boost immunization. These data show that heterologous rAd prime-boost regimens induce NAbs against the priming and boosting vectors as well as cross-reactive and anamnestic NAbs against highly related vectors.

Immunogenicity of heterologous rAd prime-boost regimens in mice with anti-Ad5 immunity. Given that the majority of individuals in the developing world have high levels of preexisting anti-Ad5 immunity (10, 15, 23, 24), we hypothesized that optimal heterologous rAd prime-boost regimens should involve two vectors that are both different from Ad5 and immunologically distinct from each other. To explore this hypothesis, we repeated the prime-boost study depicted in Fig. 2A with mice with preexisting anti-Ad5 immunity. C57BL/6 mice (n = 4/group) were preimmunized with two injections of 10^{10} vp rAd5-Empty separated by a 4-week interval, which induced Ad5-specific NAb titers of 8,192 to 16,384 (3, 11, 12, 14). Four weeks later, these mice were primed with 10^9 vp rAd35-Gag and boosted with 10^9 vp of rAd5-Gag, rAd11-Gag, rAd35-Gag, or rAd49-Gag. As shown in Fig. 3A, the rAd35/rAd49 regimen proved optimal in mice with anti-Ad5 immunity. The rAd35/rAd11 regimen was less immunogenic, presumably as a result of cross-reactive vector-specific NAbs. The rAd5-Gag vector was not an effective boosting vector as a result of preexisting anti-Ad5 immunity, and the rAd35-Gag vector was not an effective boosting vector as a result of anti-Ad35 immunity generated by the priming immunization. As depicted in Fig. 3B, these findings were confirmed by pooled peptide IFN-γ ELISPOT assays in response to a Gag peptide pool as well as the $CD8^+$ T-lymphocyte epitopes AL11 (AAVKNWMTQTL) and KV9 (KSLYNTVCV) and the $CD4^+$ T-lymphocyte epitope DD13 (DRFYKSLRAEQTD) (3). These data demonstrate that optimal heterologous rAd prime-boost regimens require two vectors that both circumvent preexisting anti-Ad5 immunity and avoid cross-reactive antivector immunity.

Vector-specific NAbs in these mice, depicted in Fig. 3C, were comparable with those observed in the previous experiment (Fig.

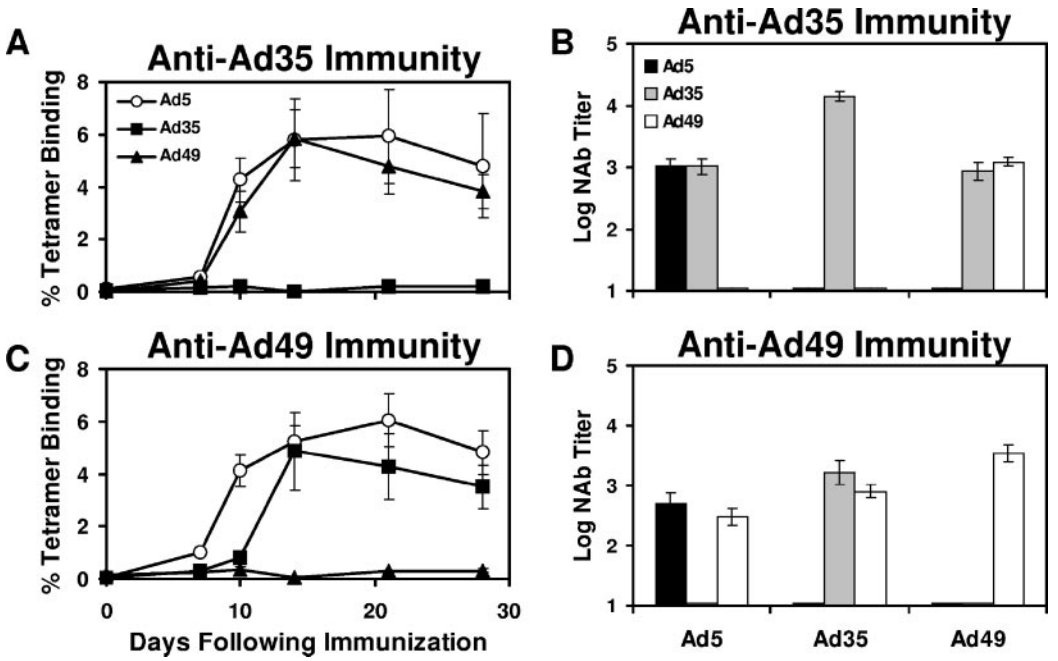

FIG. 4. Immunogenicity of rAd vectors in the presence of anti-Ad35 or anti-Ad49 immunity. Mice with (A and B) anti-Ad35 immunity or (C and D) anti-Ad49 immunity (n = 4/group) were immunized with 10^9 vp rAd5-Gag, rAd35-Gag, or rAd49-Gag. (A and C) Gag-specific cellular immune responses were assessed by D^b/AL11 tetramer binding assays at multiple time points following immunization. (B and D) Ad5-, Ad35-, and Ad49-specific NAb titers were evaluated for these mice at week 4.

2E) except that all animals had high titers of Ad5-specific NAbs. In particular, mice primed with rAd35 and boosted with rAd11 developed both Ad11-specific NAbs and anamnestic Ad35-specific NAbs following the boost immunization, confirming our previous results. Interestingly, all mice with anti-Ad5 immunity that were primed with rAd35-Gag developed slightly higher Ad35-specific NAbs than similarly vaccinated naïve mice (Fig. 2E), suggesting perhaps a subtle degree of immunologic cross-reactivity between Ad5 and Ad35 (3).

Immunogenicity of rAd vectors in the presence of anti-Ad35 or anti-Ad49 immunity. To generalize the observations regarding the suppressive effects of anti-Ad immunity, we evaluated the impact of preexisting anti-Ad35 and anti-Ad49 immunity on the immunogenicity of homologous and heterologous rAd-Gag vaccine vectors. C57BL/6 mice (n = 4/group) were preimmunized with 10^{10} vp rAd35-Empty or rAd49-Empty, and after 4 weeks these animals were vaccinated with 10^9 vp rAd5-Gag, rAd35-Gag, or rAd49-Gag. As demonstrated in Fig. 4, preexisting anti-Ad35 immunity suppressed rAd35-Gag but not rAd5-Gag or rAd49-Gag. Similarly, preexisting anti-Ad49 immunity suppressed rAd49-Gag but not rAd5-Gag or rAd35-Gag. NAb titers for these mice demonstrated the expected patterns of preexisting and vaccine-elicited vector-specific NAbs. These data confirm a lack of functionally relevant immunologic cross-reactivity among Ad5, Ad35, and Ad49.

Contribution of fiber-specific antivector immunity. As shown in Table 1, vector-specific cross-reactive NAbs correlated most closely with sequence homology in the hexon HVRs (Table 1). To determine if fiber knob-specific immunity also contributed to the suppression of these rAd vectors, we preimmunized mice with 10^{10} vp rAd35, a chimeric rAd35 vector containing the Ad5 fiber knob (rAd35k5) (12), or a similarly designed chimeric rAd35 vector containing the Ad49 fiber knob (rAd35k49). After 4 weeks, these mice were vaccinated with 10^9 vp rAd5-Gag, rAd35-Gag, or rAd49-Gag. As we observed in the previous experiment (Fig. 4A), preimmunization with rAd35 suppressed responses to rAd35-Gag but not to rAd5-Gag or rAd49-Gag (data not shown). As depicted in Fig. 5, preimmunization with rAd35k5 or rAd35k49 similarly suppressed responses to rAd35-Gag but not to rAd5-Gag or rAd49-Gag. These data demonstrate that fiber knob-specific immunity did not play a major role in suppressing vaccine-elicited immune responses in this system.

DISCUSSION

Accumulating evidence has shown that preexisting anti-Ad immunity substantially suppresses the immunogenicity of rAd vaccine vectors derived from the homologous serotype (3, 4, 11, 15). The relevance of low levels of cross-reactive vector-specific NAbs among highly related rAd vectors, however, has not previously been clearly defined. In this study, we demonstrate that cross-reactive vector-specific NAbs elicited by rAd35 and rAd11 correlated with substantial sequence homology in the hexon HVRs and suppressed the immunogenicity of heterologous rAd prime-boost regimens. In contrast, vaccine regimens that involved rAd35 and rAd49 avoided the generation of detectable cross-reactive vector-specific NAbs and proved more immunogenic than regimens that involved rAd35 and rAd11. These data demonstrate that optimal heterologous rAd prime-boost regimens require two vectors that not only circumvent preexisting antivector immunity but also avoid cross-reactive antivector immunity.

Previous studies from our laboratory and others have shown

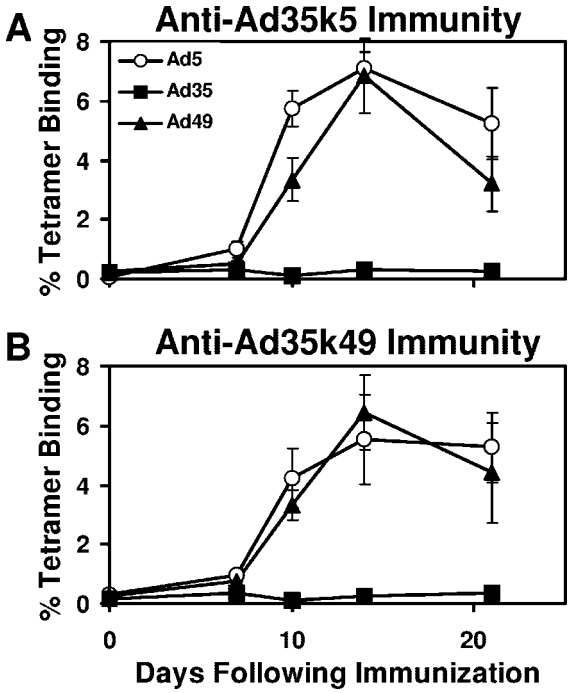

FIG. 5. Contribution of fiber-specific antivector immunity. Mice with (A) anti-Ad35k5 immunity or (B) anti-Ad35k49 immunity (n = 4/group) were immunized with 10^9 vp rAd5-Gag, rAd35-Gag, or rAd49-Gag. Gag-specific cellular immune responses were assessed by D^b/AL11 tetramer binding assays at multiple time points following immunization.

that heterologous rAd prime-boost regimens that involved rAd5 and either a rare-serotype rAd vector or a nonhuman rAd vector were also highly immunogenic (11, 13). Given the high prevalence of preexisting anti-Ad5 immunity, however, these regimens will likely be suppressed substantially in human populations in the developing world. The present studies extend these prior observations by demonstrating for the first time that heterologous rAd prime-boost regimens should optimally involve two rAd vectors that evade anti-Ad5 immunity and that are sufficiently immunologically distinct to avoid cross-reactive vector-specific NAbs. Such regimens are highly immunogenic both in the presence and in the absence of anti-Ad5 immunity and could therefore be explored further as candidate vaccine strategies. Whether utilizing rAd vectors derived from different Ad subgroups will be sufficient to avoid cross-reactive NAbs in general, however, remains to be determined.

Cross-reactive vector-specific NAb titers between rAd35 and rAd11 were 1.5 to 2.0 log lower than NAb titers against the homologous vector (Fig. 1E and F). In addition to the clearly detectable cross-reactive NAbs, mice primed with rAd35 and boosted with rAd11 developed remarkably potent anamnestic Ad35-specific NAbs following the boost immunization (Fig. 2E and 3C). In fact, mice that received the heterologous rAd35/rAd11 regimen generated higher Ad35-specific NAbs than mice that received the homologous rAd35/rAd35 regimen. These data suggest that highly related rAd vectors may be suppressed both by low-titer cross-reactive NAbs that are present at the time of immunization and by high-titer cross-reactive anamnestic NAbs that develop rapidly following immunization.

Comparisons of sequence homology among rAd vectors demonstrated 66% hexon HVR homology between the cross-reactive vectors rAd35 and rAd11 but only 15 to 27% hexon HVR homology between vectors that did not elicit cross-reactive NAbs (Table 1). These data are consistent with our model in which the hexon HVRs represent the major targets of vector-specific NAbs. We have shown previously that Ad5-specific NAbs were directed primarily against the Ad5 hexon protein (23) and that hexon HVR-chimeric rAd5 vectors effectively evaded anti-Ad5 immunity (14). The present studies extend these prior observations by showing that the hexon HVRs are also the major targets of cross-reactive NAbs among rare-serotype rAd vectors. In fact, we suspect that the degree of hexon HVR homology may prove a key factor in determining the extent of vector-specific cross-reactive NAbs. It is clear that partial hexon HVR homology is sufficient to elicit cross-reactive NAbs, although further studies will be required to determine the precise relationship between hexon HVR sequence homology and vector cross-reactivity. In contrast, fiber-specific immunity appears to play a minimal role in suppressing the immunogenicity of rAd vaccine vectors (Fig. 5). A limitation of this study, however, is that we did not explicitly evaluate the contribution of vector-specific cellular immune responses, although previous adoptive-transfer studies with mice have demonstrated that vector-specific NAbs play the dominant role in suppressing rAd vector-based vaccines (22).

These data demonstrate that the following parameters are critical for optimizing the immunogenicity of heterologous rAd prime-boost regimens: maximizing the intrinsic potency of each vector, minimizing the extent of preexisting antivector immunity, and minimizing the degree of cross-reactive antivector immunity. We propose that two rare-serotype or chimeric rAd vectors that effectively evade anti-Ad5 immunity and that have minimal hexon HVR homology will prove optimal for vaccine applications. Future studies should therefore explore the utility of these optimized heterologous rAd prime-boost vaccine regimens for pathogens such as human immunodeficiency virus type 1 that are endemic in the developing world.

ACKNOWLEDGMENTS

We thank Lennart Holterman, Ronald Vogels, Darci Gorgone, Andrew Bates, Peter Abbink, Anjali Nanda, Diane Roberts, Michelle Lifton, and Norman Letvin for generous advice, assistance, and reagents. The SIV Gag overlapping peptides were obtained from the NIH AIDS Research and Reference Reagent Program.

We acknowledge support from NIH grants AI066305 (D.H.B.), AI066924 (D.H.B.), and P30 AI060354.

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