Adenovirus Serotype 5-Specific Neutralizing Antibodies Target Multiple Hexon Hypervariable Regions

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The immunogenicity of adenovirus serotype 5 (Ad5) vectors has been shown to be suppressed by neutralizing antibodies (NAbs) directed primarily against the hexon hypervariable regions (HVRs). We previously reported that replacing all seven HVRs with those from the rare serotype virus Ad48 resulted in a chimeric Ad5HVR48(1-7) vector that largely evaded preexisting Ad5 immunity in mice and rhesus monkeys. In this study, we evaluated the extent to which Ad5-specific NAbs are directed against various HVRs. We constructed partial HVR-chimeric Ad5 vectors with only a subset of HVRs exchanged, and we utilized these vectors in both NAb assays and murine immunogenicity studies with and without baseline Ad5 immunity. Our results demonstrate that Ad5-specific NAbs target multiple HVRs, suggesting that replacing all HVRs is required to optimize evasion of anti-Ad5 immunity. These data have important implications for the development of novel vectors for both vaccines and gene therapy.

The immunogenicity of adenovirus serotype 5 (Ad5) vectors has been shown to be suppressed by neutralizing antibodies (NAbs) directed primarily against the hexon hypervariable regions (HVRs). We previously reported that replacing all seven HVRs with those from the rare serotype virus Ad48 resulted in a chimeric Ad5HVR48(1-7) vector that largely evaded preexisting Ad5 immunity in mice and rhesus monkeys. In this study, we evaluated the extent to which Ad5-specific NAbs are directed against various HVRs. We constructed partial HVR-chimeric Ad5 vectors with only a subset of HVRs exchanged, and we utilized these vectors in both NAb assays and murine immunogenicity studies with and without baseline Ad5 immunity. Our results demonstrate that Ad5-specific NAbs target multiple HVRs, suggesting that replacing all HVRs is required to optimize evasion of anti-Ad5 immunity. These data have important implications for the development of novel vectors for both vaccines and gene therapy.

Recombinant, replication-incompetent adenovirus serotype 5 (Ad5) is a highly immunogenic vector that elicits vigorous immune responses against foreign transgenes from multiple pathogens (17, 18). However, preexisting Ad5 immunity in human populations has been shown to suppress the immunogenicity of Ad5-based vectors in both preclinical studies (13, 16, 19, 20) and clinical trials (3, 11). The majority of Ad5-specific neutralizing antibodies (NAbs) are directed primarily against the hexon protein, although NAB responses have also been reported against the fiber protein (7, 8, 19, 22). Each virion consists of 240 hexon homotrimers (14), and recently determined high-resolution cryo-electron microscopy (cryo-EM) and X-ray crystal structures reveal interhexon associations as well as interactions with minor capsid proteins (9, 12). Sequence variability among adenovirus serotypes is concentrated in the seven loops located at the solvent-exposed surface of the hexon, termed hypervariable regions (HVRs) (5), which have been shown to harbor major neutralizing determinants (19, 22).

We previously reported that replacing all seven hexon HVRs in Ad5 with those from a rare human adenovirus serotype, Ad48, resulted in a chimeric vector, Ad5HVR48(1-7), that evaded the majority of preexisting Ad5 immunity in preclinical studies in mice and rhesus monkeys (13). However, the relative importance of the seven individual HVRs as NAB epitopes remain incompletely understood, and recent studies have suggested that Ad5 NAB responses may actually be focused primarily on one specific HVR, such as HVR1 or HVR5 (1, 15). In this study, we characterized the contribution of individual hexon HVRs as Ad5 NAB epitopes. We constructed chimeric Ad5 vectors in which only subsets of HVRs were exchanged and evaluated these vectors in both NAB assays and immunogenicity studies.

We first designed the chimeric vectors Ad5HVR48(1-6), Ad5HVR48(1-3), Ad5HVR48(2-7), and Ad5HVR48(4-7), in which the specific Ad5 HVRs that have been exchanged with the corresponding regions from Ad48 are designated in parentheses (Fig. 1A). Chimeric Ad5 hexon genes containing these HVRs were generated by PCR amplification and cloned into the Ad5 cosmid pWE.Ad5.Aflii-rITR.dE3. Replication-incompetent Ad5 vectors with E1/E3 deleted and containing chimeric hexon genes were produced essentially as described previously (20).

The Ad5HVR48(1-6), Ad5HVR48(2-7), and Ad5HVR48(4-7) vectors proved nonviable. In contrast, the Ad5HVR48(1-3) vector was viable and could be produced to high titers (data not shown). To evaluate the contributions of HVR1, HVR2, and HVR3 as NAB epitopes, we determined NAB responses against Ad5, Ad5HVR48(1-3), Ad5HVR48(1-7), and Ad48 expressing luciferase as described previously (17) in both murine and human serum samples. All vectors were analytically similar in terms of titer, specific infectivity, transgene expression, and dynamic range in the NAB assays. C57BL/6 mice (n = 72) were preimmunized with two injections of 10{sup 10} virus particles (vp) of Ad5-Empty to generate Ad5 immunity, and serum samples were analyzed for Ad5 NAB titers (Fig. 1B). As expected, high Ad5 NAB titers (median log titer, 3.9) were detected in all the vaccinated mice, and Ad48 NAB titers were not observed. Intermediate NAB titers were evident against the chimeric vectors Ad5HVR48(1-3) and Ad5HVR48(1-7). Median Ad5HVR48(1-7) NAB titers (median log titer, 2.4) were 1.5 log lower than median Ad5 NAB titers (median log titer, 3.9; P < 0.0001, Wilcoxon signed-rank test) (Fig. 1B), similar to our previous findings (13). Ad5HVR48(1-3) NAB titers (median log titer, 2.9) proved 1.0 log lower than Ad5 NAB titers (P < 0.0001) but 0.5 log higher than Ad5HVR48(1-7) median NAB titers (P < 0.0001) (Fig. 1B). These data indicate that Ad5 NABs are directed partially, but not exclusively, against HVR1 to HVR3 in mice. The stepwise evasion of Ad5 NABs by the partial chimera Ad5HVR48(1-3) suggests that dominant NABs are not in fact directed exclusively against one specific HVR but, rather, likely target epitopes in both HVR1 to HVR3 and HVR4 to HVR7.

We next evaluated NAB responses against Ad5, Ad5HVR48(1-3), Ad5HVR48(1-7), and Ad48 in sera from 267 healthy adults from South Africa (2) (Fig. 1C). High Ad5 NAB titers were detected in these samples (median log titer, 3.0) as expected, indic
ative of natural Ad5 exposure, and Ad48 NAb titers were low (2). Ad5HVR48(1-3) NAb titers (median log titer, 2.0) were 0.9 log lower than Ad5 NAb titers (P < 0.0001) (Fig. 1C), indicating that approximately 90% of Ad5 NAb activity was directed against epitopes located in the seven hexon HVRs. Ad5HVR48(1-3) NAb titers (median log titer, 2.4) were 0.5 log lower than Ad5 titers (P < 0.0001) but 0.5 log higher than Ad5HVR48(1-7) NAb titers (P < 0.0016) (Fig. 1C). Thus, swapping just HVR1 to HVR3 does not fully recapitulate the phenotype of Ad5HVR48(1-7). These data confirm our observations with mice and suggest that Ad5 NAbs are directed against both HVR1 to HVR3 and HVR4 to HVR7 in humans.

We explored the immunogenicity of the novel Ad5HVR48(1-3)-simian immunodeficiency virus (SIV) Gag vector in C57BL/6 mice to evaluate if swapping just HVR1 to HVR3 would be sufficient to evade high levels of anti-Ad5 immunity in vivo. To induce high levels of anti-Ad5 immunity, we preimmunized mice intramuscularly (i.m.) twice at a 4-week interval with 10^10 vp of Ad5-Empty in 100 μl of sterile phosphate-buffered saline (PBS) as described previously (10). Naïve as well as Ad5-preimmunized C57BL/6 mice (n = 8/group) were then i.m. immunized once with 10^9 vp of Ad5-SIV Gag, Ad5HVR48(1-7)-SIV Gag, Ad5HVR48(1-3)-SIV Gag, and Ad5HVR48(1-7)-SIV Gag. Tetrameric H-2Db complexes folded around the immunodominant SIV Gag AL11 epitope (AAVKNWMTQTL) (10) were prepared and used to measure SIV Gag-specific CD8^+ T-lymphocyte responses on days 0, 7, 14, and 21 following immunization. CD8^+ T lymphocytes from naïve mice exhibited <0.1% tetramer staining. In naïve mice, the kinetics and magnitude of AL11-specific CD8^+ T-lymphocyte responses elicited by Ad5HVR48(1-3)-SIV Gag were comparable to responses elicited by Ad5-SIV Gag, Ad5HVR48(1-7)-SIV Gag, and Ad5HVR48(1-7)-SIV Gag (Fig. 2A). To evaluate functional responses, we utilized splenocytes from day 28 in gamma interferon (IFN-γ) enzyme-linked immunosorbent spot (ELISPOT) and intracellular cytokine-staining (ICS) assays as described previously (10). IFN-γ ELISPOT responses to overlapping Gag peptides, the dominant CD8^+ T cell epitope AL11 (AAVKNWMTQTL), the subdominant CD8^+ T epitope KV9 (KSLYNTVCV), and the CD4^+ T cell epitope DD13 (DRFYKSLRAEQTD) (10) were comparable among all four vectors (Fig. 2A). IFN-γ ICS responses also proved similar among these vectors (Fig. 2A), and cytokine secretion and memory subpopulation phenotypes were also comparable (data not shown).

In mice with high baseline Ad5 NAb titers (median log NAb titer, 4.0), which model the upper bound of Ad5 NAb titers in Africa (2), the immunogenicity of Ad5-SIV Gag was abrogated, as expected, while the immunogenicity of Ad5HVR48(1-7)-SIV Gag was largely preserved (Fig. 2B), consistent with our previously reported findings (13). Importantly, the immunogenicity of both Ad5HVR48(1-3)-SIV Gag and Ad5HVR48(1-7)-SIV Gag was also
suppressed (Fig. 2B). These data indicate that exchanging just HVR1 to HVR3 was insufficient to circumvent high levels of preexisting Ad5 immunity, consistent with the intermediate reduction of NAb titers to the Ad5HVR48(1-3) vector in virus neutralization assays in vitro (Fig. 1B and C). These data suggest that Ad5 NAb epitopes that are relevant in vivo for suppression of vector immunogenicity likely include multiple HVRs and that Ad5HVR48(1-3) does not adequately evade Ad5 NAb responses in vivo.

To evaluate in greater detail the role of HVR4 to HVR7 as neutralization determinants, we attempted to produce the Ad5HVR48(4-7) vector, but this vector proved nonviable (Fig. 1A). We then attempted to generate Ad5HVR48(4), Ad5HVR48(5), Ad5HVR48(6), and Ad5HVR48(7) vectors in which only single HVRs were exchanged (Fig. 3A). Of these, only the Ad5HVR48(4) and Ad5HVR48(5) vectors could be produced to high titers (data not shown). We evaluated the immunogenicity of Ad5HVR48(4)-SIV Gag and Ad5HVR48(5)-SIV Gag vectors in studies similar to those described in the legend to Fig. 2. Naïve and Ad5-preimmunized C57BL/6 mice (n = 8/group) were immunized i.m. with 10⁹ vp of Ad5, Ad5HVR48(1), Ad5HVR48(1-7), and Ad5HVR48(1-3) expressing SIV Gag. AL11-specific tetramer responses at multiple time points, IFN-γ ELISpot assays using splenocytes on day 28, and ICS assays using splenocytes on day 28 were utilized to measure SIV Gag-specific cellular immune responses. SFC, spot-forming cells per 10⁷ splenocytes.

To investigate further the immunogenicity of chimeric Ad5 vectors with all seven HVRs exchanged, we designed a series of vectors similar to Ad5HVR48(1-7) (13) but using HVRs from Ad11, Ad26, Ad35, Ad49, and AdC68. The Ad5HVR11(1-7), Ad5HVR26(1-7), Ad5HVR35(1-7), and Ad5HVR49(1-7) vectors proved nonviable, but the Ad5HVRC68(1-7) vectors were successfully generated (Fig. 4A). The Ad5HVRC68(1-7) and Ad5HVR48(1-7) vectors expressing SIV Gag proved comparably immunogenic in naïve mice (Fig. 4B). Mice with high levels of anti-Ad5 immunity were then primed at day 0 and boosted at day 28 with the following vectors expressing SIV Gag (n = 8/group): Ad5/Ad5, Ad5HVRC68(1-7)/Ad5, Ad5HVRC68(1-7)/Ad5HVR48(1-7), and Ad5HVRC68(1-7)/Ad5HVRC68(1-7). Following the priming immunization, the Ad5-SIV Gag vector was suppressed, whereas the Ad5HVRC68(1-7)-SIV Gag vector remained highly immunogenic, demonstrating that this vector evaded anti-Ad5 immunity (Fig. 4C) in a manner similar to that of Ad5HVR48(1-7). Interestingly, following the
booster immunization, the heterologous Ad5HVR C68(1-7)/ Ad5HVR48(1-7) regimen proved the most immunogenic, presumably because these two vectors had distinct HVRs and thus were serologically distinct (Fig. 4C). In contrast, the Ad5 booster was suppressed as a result of the preexisting Ad5 immunity, and the Ad5HVR C68(1-7) booster was suppressed as a result of antivector immunity elicited by the priming immunization. These data confirm that Ad5HVR C68(1-7) is a potent vector that effectively evades anti-Ad5 immunity and is sufficiently non-cross-reactive with Ad5HVR48(1-7) to allow the development of potent heterologous prime-boost regimens.

The seroprevalence of AdC68 has been reported to be low in humans (4, 6, 21).

Ad5-specific antibodies have been shown to be directed against multiple capsid proteins, including fiber, penton, and hexon (8, 19), but dominant NAb responses that are critical for suppressing Ad5 vectors appear to be directed largely against the hexon HVRs (19, 22). However, the precise targets of Ad5 NAbs remain unclear. In the present study, we assessed the contributions of the seven HVRs as targets for Ad5 NAbs. We built on prior studies from our group that showed that an Ad5HVR48(1-7) vector in which all seven HVRs were exchanged with those from the rare...
serotype vector Ad48 largely evaded Ad5 immunity in both mice and rhesus monkeys (13). In the present study, we observed that swapping only HVR1 to HVR3, HVR4, or HVR5 individually was insufficient to evade preexisting Ad5-specific immunity in mice, whereas swapping all seven HVRs with those from the coxsackievirus and adenovirus receptor (CAR)-binding virus AdC68 recapitulated the phenotype observed with Ad5HVR48(1-7). Consistent with these results, NAb titers to Ad5HVR48(1-3) were significantly lower than those to Ad5 but were higher than those to Ad5HVR48(1-7) in sera from vaccinated mice and humans with natural Ad5 immunity. These data indicate that Ad5-specific NAbs target multiple HVRs, including epitopes located in both HVR1 to HVR3 and HVR4 to HVR7. Thus, swapping all HVRs simultaneously is likely required for optimal evasion of antivector immunity.

Our data contrast with two previous studies that reported substantial evasion of Ad5 immunity by Ad5 vectors in which single HVRs (HVR1, HVR5) were mutated (1, 15). In our studies, swapping just HVR1 (Fig. 2B) or HVR5 (Fig. 3C) failed to evade high levels of Ad5 NAbs, suggesting important limitations of this approach. A major difference is that these prior reports utilized lower Ad5 NAb titers and higher doses of the vectors than our studies. Thus, it is possible that single HVR swaps may evade relatively low levels of Ad5 NAb titers if high doses of the vectors are utilized, whereas complete HVR swaps appear to be required for circumventing high levels of preexisting Ad5 immunity.
A technical challenge in the present study was the fact that many chimeric vectors proved nonviable. Recent studies have reported the crystal and cryo-EM structures of the Ad5 capsid at 3.5-Å resolution, providing a detailed picture of the structural constraints of the hexon HVRs, including substantial contacts with minor capsid proteins (9, 12). These data suggest that there are both structural and biochemical constraints on the HVRs that limit the ability to manipulate these regions, and high-throughput methods to determine the viability of HVR-exchanged Ad vectors are lacking. Development of such techniques would accelerate the generation of HVR-chimeric vectors and prime-boost regimens.

In summary, our findings indicate that multiple HVRs are targeted by both natural and vaccine-elicited Ad5 NAbs and that vectors with complete HVR swaps outperform those with partial HVR swaps in terms of evasion of vector-specific NAbs. Moreover, we show that a novel Ad5HVRC68(1-7) vector recapitulates the ability of Ad5HV48(1-7) to evade Ad5 immunity in mice. These findings add to our understanding of the humoral immune response to adenoviruses and contribute to our understanding of capsid-chimeric vectors and prime-boost regimens for both vaccine development and gene therapy.

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