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# Neutralizing Antibodies to Adenovirus Serotype 5 Vaccine Vectors Are Directed Primarily against the Adenovirus Hexon Protein[1]

**Shawn M. Sumida,* Diana M. Truitt,* Angelique A. C. Lemckert,† Ronald Vogels,† Jerome H. H. V. Custers,† Marylyn M. Addo,‡ Shahin Lockman,§ Trevor Peter,§ Fred W. Peyerl,* Michael G. Kishko,* Shawn S. Jackson,* Darci A. Gorgone,* Michelle A. Lifton,* Myron Essex,§ Bruce D. Walker,‡ Jaap Goudsmit,† Menzo J. E. Havenga,† and Dan H. Barouch[2]***

**The utility of recombinant adenovirus serotype 5 (rAd5) vector-based vaccines for HIV-1 and other pathogens will likely be limited by the high prevalence of pre-existing Ad5-specific neutralizing Abs (NAbs) in human populations. However, the immunodominant targets of Ad5-specific NAbs in humans remain poorly characterized. In this study, we assess the titers and primary determinants of Ad5-specific NAbs in individuals from both the United States and the developing world. Importantly, median Ad5-specific NAb titers were >10-fold higher in sub-Saharan Africa compared with the United States. Moreover, hexon-specific NAb titers were 4- to 10-fold higher than fiber-specific NAb titers in these cohorts by virus neutralization assays using capsid chimeric viruses. We next performed adoptive transfer studies in mice to evaluate the functional capacity of hexon- and fiber-specific NAbs to suppress the immunogenicity of a prototype rAd5-Env vaccine. Hexon-specific NAbs were remarkably efficient at suppressing Env-specific immune responses elicited by the rAd5 vaccine. In contrast, fiber-specific NAbs exerted only minimal suppressive effects on rAd5 vaccine immunogenicity. These data demonstrate that functionally significant Ad5-specific NAbs are directed primarily against the Ad5 hexon protein in both humans and mice. These studies suggest a potential strategy for engineering novel Ad5 vectors to evade dominant Ad5-specific NAbs.** ***The Journal of Immunology,*** **2005, 174: 7179–7185.**

Recombinant adenovirus serotype 5 (rAd5)[3] vector-based vaccines have been shown to elicit potent and protective immune responses in a variety of animal models (1–3). These vaccines are therefore being advanced into large-scale clinical trials for both HIV-1 and other pathogens (4–6). A major limitation of this approach, however, is the high prevalence of pre-existing anti-Ad5 immunity in human populations. Anti-Ad5 immunity has been shown to suppress substantially the immunogenicity of rAd5 vaccines in studies in mice (7–10), rhesus monkeys (11), and humans in phase 1 clinical trials (12).

Developing novel Ad vectors that can circumvent the suppressive effects of pre-existing anti-Ad5 immunity is therefore an important research priority. One promising strategy involves the development of vectors from novel Ad serotypes. For example, we have shown that rAd serotype 35 (rAd35) vector-based vaccines elicited potent cellular immune responses that were not significantly suppressed by anti-Ad5 immunity (8, 13). Similarly, chimpanzee Ads have been shown to elicit immune responses that were minimally affected by anti-Ad5 immunity (14–17). However, far less is known about the pathogenesis, disease associations, safety, and manufacturing capacity of these novel Ad serotypes compared with Ad5.

An alternative strategy is to engineer rAd5 vectors to evade dominant Ad5-specific immune responses. However, our understanding of anti-Ad5 immunity remains incomplete. We have recently shown that neutralizing Abs (NAbs) and $CD8^+$ T lymphocyte responses both contribute to anti-Ad5 immunity, although Ad5-specific NAbs appear to play the primary role (9). A detailed understanding of the immunodominant targets of Ad5-specific NAbs will therefore be critical for the development of improved rAd5 vectors.

The Ad capsid consists of three major structural proteins: hexon, penton, and fiber. A total of 240 hexon capsomeres forms the majority of the capsid structure, whereas 12 vertex capsomeres consist of the fiber attachment proteins and their penton bases. Hexon-specific Abs have been shown by several laboratories to exert significant neutralizing activity in vitro (18, 19). Moreover, studies in rodents using hexon-chimeric viruses have suggested that hexon-specific immune responses also contribute substantially to anti-Ad5 immunity in vivo (20–22). In contrast, recent clinical studies have reported that fiber- and penton-specific Abs were more frequent and higher titer than hexon-specific Abs in humans (23) and that fiber- and penton-specific Abs exerted synergistic

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[3] Abbreviations used in this paper: rAd5, recombinant adenovirus serotype 5; NAb, neutralizing Ab; vp, virus particle.

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neutralizing activity (24). It is clear from these studies that Ad5-specific Abs against multiple capsid components are generated. However, the relative significance of hexon-, fiber-, and penton-specific Abs in suppressing rAd5 vaccine immunogenicity has not been fully characterized.

In this study, we assess NAb titers against the major Ad5 capsid proteins in human serum samples from both the United States and the developing world. We also investigate the functional significance of hexon- and fiber-specific NAbs to suppress the immunogenicity of a prototype rAd5-Env vaccine in mice using adoptive transfer studies. These studies demonstrate that Ad5-specific NAbs are directed primarily against the Ad5 hexon protein.

## Materials and Methods

### *Human serum samples*

Serum samples from clinical diagnostic specimens were obtained from healthy adults from the United States ($n = 59$), Haiti ($n = 67$), Botswana ($n = 57$), Zambia ($n = 29$), and South Africa ($n = 59$) without patient identifiers. Studies using these samples were approved by our Institutional Review Board.

### *Virus neutralization assay*

Ad-specific NAb responses were assessed by luciferase-based virus neutralization assays essentially as described (25). A549 human lung carcinoma cells were plated at a density of $1 \times 10^4$ cells per well in 96-well plates and infected with E1-deleted, replication-incompetent rAd-Luciferase reporter constructs at a multiplicity of infection of 500 with 2-fold serial dilutions of serum in 200-$\mu$l reaction volumes. Following a 24-h incubation, luciferase activity in the cells was measured using the Steady-Glo Luciferase Reagent System (Promega). Ninety percent neutralization titers were defined as the maximum serum dilution that neutralized 90% of luciferase activity.

### *Mice and immunizations*

Six- to 8-wk-old BALB/c or C57BL/6 mice were purchased from Charles River Laboratories. For rAd immunizations, mice were injected i.m. with varying doses of E1-deleted, replication-incompetent rAd vectors in 100 $\mu$l of sterile PBS. All animal studies were approved by our Institutional Animal Care and Use Committee.

### *Adoptive transfers*

To study the inhibitory effects of Ad5-specific NAbs, adoptive transfer studies were performed essentially as described (9). Mice were preimmunized with capsid chimeric rAd5/rAd35 vectors, and purified IgG was prepared from 2 ml of serum pooled from eight mice using a Protein A Ab Purification kit (Sigma-Aldrich) and dialyzed into 5 ml of endotoxin-free Dulbecco's PBS (Invitrogen Life Technologies) before use in adoptive transfer studies. These IgG preparations had similar concentrations of 2–3 mg/ml total IgG (Bio-Rad). A volume of 500 $\mu$l of purified IgG was then adoptively transferred to naive recipient mice by tail vein injection. On the day following adoptive transfer, recipient mice were immunized with $10^8$ virus particles (vp) of rAd5-Env.

### *Western blots*

Western blots were performed to characterize Ad-specific NAbs in purified IgG preparations. A total of $10^8$ vp of rAd5 or rAd35 was electrophoresed by SDS-PAGE using a 7.5% resolving gel (Bio-Rad) and transferred to nitrocellulose membranes for Western blotting. Membranes were blocked overnight in TTBS (0.05% Tween 20, 50 mM Tris-HCl (pH 7.5), 150 mM NaCl) containing 2.5% nonfat dry milk. Membranes were then incubated with purified mouse IgG at a 1/100 dilution for 2 h in TTBS containing 0.5% milk, washed three times with TTBS containing 0.5% milk, and incubated with ImmunoPure peroxidase-conjugated goat anti-mouse IgG secondary Ab (Pierce) at a 1/20,000 dilution for 1 h in TTBS containing 0.5% milk. Samples were detected with the SuperSignal West Pico Mouse IgG Detection kit (Pierce) following the manufacturer's protocol and exposed to BioMax ML film (Kodak) for 2 min.

### *Tetramer staining*

Tetrameric H-2D$^d$ complexes folded around the dominant HIV-1 IIIB V3 loop P18 epitope peptide (P18-I10; RGPGRAFVTI) (26) were prepared and used to stain P18-specific CD8$^+$ T lymphocytes from BALB/c mice essentially as described (27, 28). Mouse blood was collected in RPMI 1640 containing 40 U/ml heparin. Following lysis of the RBCs, 0.1 $\mu$g of PE-labeled D$^d$/P18 tetramer in conjunction with allophycocyanin-labeled anti-CD8$\alpha$ mAb (Ly-2; Caltag) was used to stain P18-specific CD8$^+$ T lymphocytes. The cells were washed in PBS containing 2% FBS and fixed in 0.5 ml of PBS containing 1.5% paraformaldehyde. Samples were analyzed by two-color flow cytometry on a FACSCalibur (BD Biosciences). Gated CD8$^+$ T lymphocytes were examined for staining with the D$^d$/P18 tetramer. CD8$^+$ T lymphocytes from naive mice were used as negative controls and exhibited $<0.1\%$ tetramer staining. For C57BL/6 mice immunized with vectors expressing SIV Gag, we used tetrameric H-2D$^b$ complexes folded around the dominant SIVmac239 Gag AL11 epitope peptide (AAVKNWMTQTL) (8).

### *ELISPOT*

ELISPOT assays were used to assess IFN-$\gamma$ production by splenocytes from BALB/c mice immunized with vectors expressing HIV-1 Env IIIB as described (7, 28). Cytokine production was assessed in response to the P18 epitope peptide or a pool of 47 overlapping 15-aa peptides derived from HIV-1 Env IIIB gp120 (Centralised Facility for AIDS Reagents, Potters Bar, U.K.). Ninety-six-well multiscreen plates (Millipore) coated overnight with 100 $\mu$l/well of 10 $\mu$g/ml rat anti-mouse IFN-$\gamma$ (BD Pharmingen) in PBS were washed with endotoxin-free Dulbecco's PBS (Invitrogen Life Technologies) containing 0.25% Tween 20 and blocked with PBS containing 5% FBS for 2 h at 37°C. The plates were washed three times with Dulbecco's PBS containing 0.25% Tween 20, rinsed with RPMI 1640 containing 10% FBS, and incubated in triplicate with $5 \times 10^5$ splenocytes per well in a 100-$\mu$l reaction volume containing 2 $\mu$g/ml peptide. For studies using the Env peptide pool, each peptide in the pool was present at 2 $\mu$g/ml. Following an 18-h incubation, the plates were washed nine times with Dulbecco's PBS containing 0.25% Tween 20 and once with distilled water. The plates were then incubated for 2 h with 75 $\mu$l/well of 5 $\mu$g/ml biotinylated rat anti-mouse IFN-$\gamma$ (BD Pharmingen), washed six times with Coulter Wash (Coulter), and incubated for 2 h with a 1/500 dilution of streptavidin-alkaline phosphatase (Southern Biotechnology Associates). Following five washes with Coulter Wash and one with PBS, the plates were developed with NBT/5-bromo-4-chloro-3-indolyl-phosphate chromogen (Pierce), stopped by washing with tap water, air dried, and read using an ELISPOT reader (Hitech Instruments).

### *ELISA*

Serum Ab titers from immunized mice specific for HIV-1 Env or SIV Gag were measured by a direct ELISA as described (7, 28). Ninety-six well plates coated overnight with 100 $\mu$l/well of 1 $\mu$g/ml recombinant HIV-1 Env IIIB gp120 or SIV Gag p27 (Intracel) in PBS were blocked for 2 h with PBS containing 2% BSA and 0.05% Tween 20. Sera were then added in serial dilutions and incubated for 1 h. The plates were washed three times with PBS containing 0.05% Tween 20 and incubated for 1 h with a 1/2000 dilution of a peroxidase-conjugated affinity-purified rabbit anti-mouse secondary Ab (Jackson ImmunoResearch Laboratories). The plates were washed three times, developed with tetramethylbenzidine (Kirkegaard & Perry Laboratories), stopped with 1% HCl, and analyzed at 450 nm with a Dynatech MR5000 ELISA plate reader.

### *Statistical analyses*

Statistical analyses were performed with GraphPad Prism version 4.01 (GraphPad Software). Comparisons of mean responses among groups were performed by ANOVA with Bonferroni adjustments to account for multiple comparisons. In all cases, $p$ values of $<0.05$ were considered significant.

## Results

### *International seroprevalence and NAb titers to Ad5, Ad35, and Ad11*

We initiated studies to assess the seroprevalence and NAb titers to Ad5 and alternate Ad serotypes in the developing world. We performed functional luciferase-based virus neutralization assays (25) using serum samples from healthy adults from the United States ($n = 59$), Haiti ($n = 67$), Botswana ($n = 57$), Zambia ($n = 29$), and South Africa ($n = 59$). Seroprevalence was defined as the percentage of serum samples that neutralized $>90\%$ luciferase activity at a serum dilution of 1/16 or greater. As shown in Fig. 1*a*, the Ad5 seroprevalence was 50% in the United States. In contrast, the Ad5 seroprevalence was 82% in Haiti, 93% in Botswana, 93%

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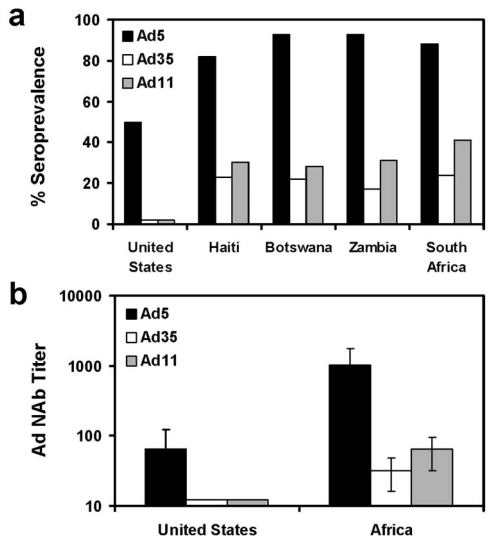


**FIGURE 1.** Ad5, Ad35, and Ad11 seroprevalence and NAb titers. Seroprevalence (*a*) and NAb titers (*b*) to rAd5, rAd35, and rAd11 as determined by luciferase-based virus neutralization assays. Serum samples were obtained from healthy adults in the United States ($n = 59$), Haiti ($n = 67$), Botswana ($n = 57$), Zambia ($n = 29$), and South Africa ($n = 59$).

in Zambia, and 88% in South Africa. These data demonstrate that the Ad5 seroprevalence was substantially higher in the developing world compared with the United States.

As shown in Fig. 1*b*, the median Ad5-specific NAb titers from sub-Saharan Africa were strikingly >10-fold higher than the median titers found in the United States ($p < 0.001$). These data extend our previous findings (13, 29) and show that Ad5-specific NAbs were nearly universal and high titer in these populations in the developing world. In contrast, the Ad35 and Ad11 seroprevalence and titers were substantially lower. These data suggest that pre-existing anti-Ad5 immunity will likely substantially suppress the immunogenicity and clinical utility of rAd5 vector-based vaccines in the developing world.

### *Immunodominant targets of Ad5-specific NAbs*

We next used these human serum samples to determine the dominant antigenic targets of Ad5-specific NAbs in both the United States ($n = 59$) and sub-Saharan Africa ($n = 145$). Given the lack of detectable NAb cross-reactivity between Ad5 and Ad35 (8), we used capsid chimeric rAd5/rAd35 viruses expressing luciferase in virus neutralization assays. These vectors incorporated various combinations of Ad5 and Ad35 hexon, penton, and fiber proteins in the context of intact viral particles with wild-type growth kinetics (30, 31). As depicted in Table I, we used the following chimeric vectors in this study: rAd5f35 (rAd5 containing the Ad35 fiber), rAd5f35p35 (rAd5 containing the Ad35 fiber and penton), and rAd35f5 (rAd35 containing the Ad5 fiber).

Table I. *Capsid composition of recombinant chimeric rAd5/rAd35 vectors*

| Vector | Hexon | Penton | Fiber |
|---|---|---|---|
| rAd5 | Ad5 | Ad5 | Ad5 |
| rAd5f35 | Ad5 | Ad5 | Ad35 |
| rAd5f35p35 | Ad5 | Ad35 | Ad35 |
| rAd35f5 | Ad35 | Ad35 | Ad5 |
| rAd35 | Ad35 | Ad35 | Ad35 |

As shown in Fig. 2*a*, comparable NAb titers were observed in human serum samples against rAd5, rAd5f35, and rAd5f35p35. Because the capsid of the rAd5f35p35 vector contained the Ad5 hexon but the Ad35 fiber and Ad35 penton, these data suggest that the majority of Ad5-specific NAb activity was directed against the Ad5 hexon. Lower but clearly detectable titers were also measured against rAd35f5, demonstrating that Ad5 fiber-specific NAbs were present but at 4- to 10-fold lower titers than Ad5 hexon-specific NAbs in these samples. We were not able to measure Ad5 penton-specific NAbs directly using this panel of capsid chimeric viruses, but the similar NAb titers against rAd5, rAd5f35, and rAd5f35p35 suggested that penton-specific NAbs as well as the combination of fiber- and penton-specific NAbs played at most a minor role in this neutralization. Thus, Ad5-specific NAbs were detected against multiple capsid components, but hexon-specific NAbs were substantially higher titer than fiber-specific NAbs in subjects from both the United States and sub-Saharan Africa. Binding Abs against all major capsid components were also detected by Western blots in these samples (data not shown), indicating that only a subset of binding Abs were in fact functionally relevant NAbs.

We next confirmed these findings using serum from experimentally immunized mice. BALB/c mice ($n = 4$/group) were immunized with $10^{10}$ vp of rAd5 or rAd35, and serum was obtained at wk 4 following immunization and was used in virus neutralization assays. As shown in Fig. 2*b*, serum from rAd5-immunized mice had comparable NAb titers to rAd5, rAd5f35, and rAd5f35p35, but exhibited 6-fold lower NAb titers to rAd35f5, consistent with the results obtained with the human samples. As expected, serum from rAd5-immunized mice had no detectable cross-reactive NAbs against rAd35 (8). Conversely, serum from rAd35-immunized mice had comparable NAb titers to rAd35 and rAd35f5, but exhibited 5-fold lower NAb titers to rAd5f35 and rAd5f35p35. These data indicate that the immunodominant targets of Ad-specific NAbs in immunized mice mirrored those observed in humans.

### *Suppressive effects of low titers of Ad-specific NAbs*

We next assessed the functional significance of NAbs against the major Ad5 capsid proteins by determining their capacity to suppress rAd5 vaccine immunogenicity following adoptive transfer in

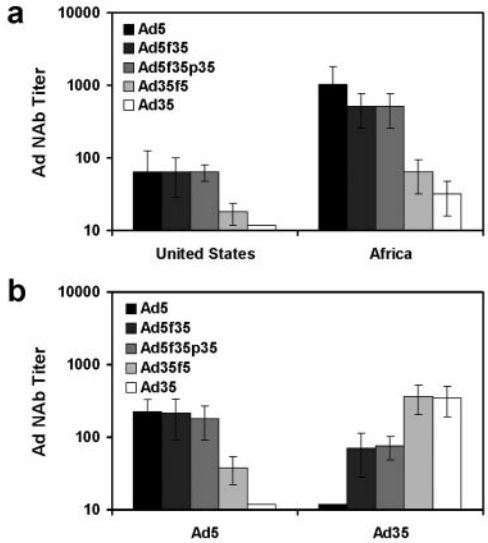


**FIGURE 2.** Immunodominant targets of Ad-specific NAbs. Serum samples from Ad5-seropositive humans (*a*) or rAd5- or rAd35-immunized mice (*b*) were assessed for NAb titers to various capsid chimeric rAd5/rAd35 viruses. NAb titers to rAd5, rAd5f35, rAd5f35p35, rAd35f5, and rAd35 were determined.

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mice. We have previously reported that anti-Ad35 immunity did not suppress rAd5 vaccine-elicited immune responses in mice (8). Therefore, we used capsid chimeric rAd5/rAd35 viruses to elicit immune responses against specific components of the Ad5 capsid. This strategy has an advantage over using soluble proteins to induce anti-Ad5 immunity in that these chimeric viruses present the various capsid proteins to the immune system in the context of intact, infectious viral particles.

Donor mice received one injection of $10^{10}$ vp of rAd5, rAd5f35p35, rAd35f5, rAd35, or saline to elicit low NAb titers, and IgG was purified from serum after 4 wk. As shown in Fig. 3*a*, we confirmed Ad5- and Ad35-specific neutralizing activity in these purified IgG preparations before adoptive transfer. As expected, IgG from mice immunized with rAd5 or rAd35 exhibited low serotype-specific NAb titers of 64–128 without detectable cross-reactivity. In contrast, IgG from mice immunized with the chimeric viruses exhibited NAb titers against both Ad5 and Ad35. IgG from mice that received rAd5f35p35 contained Ad5-specific NAbs that were presumably directed against the Ad5 hexon as well as low-titer Ad35-specific NAbs that were presumably directed against the Ad35 penton and fiber. Similarly, IgG from mice that received rAd35f5 contained Ad35-specific NAbs against the Ad35 hexon and penton as well as low-titer Ad5-specific NAbs that were presumably directed against the Ad5 fiber.

Adoptive transfer studies were then performed to assess the ability of these IgG preparations containing Ad-specific NAbs to suppress rAd5 vaccine immunogenicity. Groups of naive recipient mice ($n$ = 4/group) received 500 $\mu$l of purified IgG by tail vein injection before immunization with $10^8$ vp of rAd5-Env. Env-specific $CD8^+$ T lymphocyte responses against the dominant P18 epitope (RGPGRAFVTI) (26) were assessed by $D^d$/P18 tetramer binding assays (27, 28). As shown in Fig. 3*b*, adoptive transfer of IgG from mice that received rAd35 or rAd35f5 (●, ■) did not detectably suppress tetramer$^+$CD8$^+$ T lymphocyte responses. In contrast, adoptive transfer of IgG purified from mice that received rAd5 or rAd5f35p35 (○, △) substantially suppressed peak tetramer$^+$CD8$^+$ T lymphocyte responses by 54% ($p < 0.01$ comparing mean tetramer responses among groups on day 10 following immunization using ANOVA with Bonferroni adjustments). There was also a trend toward a 12–37% reduction of memory responses on day 28 following immunization. Thus, even low titers of hexon-specific NAbs effectively suppressed peak rAd5 vaccine-elicited immune responses.

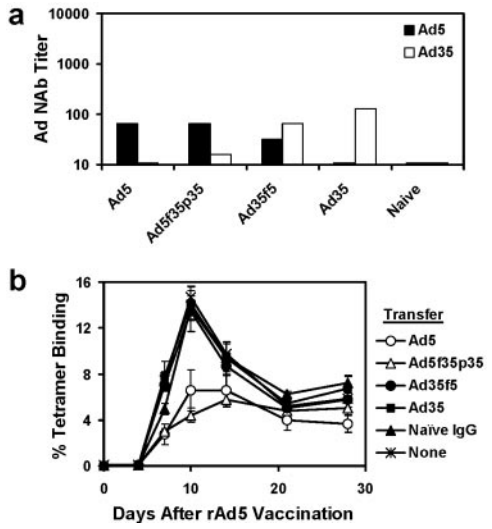


**FIGURE 3.** Adoptive transfer studies using low titers of Ad-specific NAbs. IgG was purified from pooled serum from BALB/c mice that received one injection of $10^{10}$ vp of rAd5, rAd5f35p35, rAd35f5, rAd35, or saline. *a*, The IgG preparations were assessed for NAbs to rAd5 and rAd35. The IgG preparations were then adoptively transferred into naive BALB/c mice ($n$ = 4/group) by tail vein injection before immunization with $10^8$ vp of rAd5-Env. *b*, Vaccine-elicited immune responses following immunization were determined by $D^d$/P18 tetramer binding assays. Controls included mice that received adoptive transfer of naive IgG purified from saline-injected mice (Naive) and mice that received no adoptive transfer (None).

### *Suppressive effects of high titers of Ad-specific NAbs*

We next examined the suppressive effects of high titers of Ad5 hexon- and fiber-specific NAbs by repeating this experiment using donor mice that were preimmunized twice with $10^{10}$ vp of rAd5, rAd5f35p35, rAd35f5, rAd35, or saline. As shown in Fig. 4*a*, purified IgG from these mice had Ad5- and Ad35-specific NAb titers that were ~100-fold higher than in the previous experiment. IgG from mice that received rAd5 or rAd35 exhibited potent, serotype-specific NAb titers of 8192. IgG from mice that received rAd5f35p35 had potent Ad5-specific NAb titers that were presumably directed against the Ad5 hexon as well as lower Ad35-specific NAb titers that were presumably directed against the Ad35 fiber and penton. Similarly, IgG from mice that received rAd35f5 exhibited potent Ad35-specific NAb titers against the Ad35 hexon and penton and lower Ad5-specific NAb titers that were presumably directed against the Ad5 fiber. The clear induction of Ad5 fiber-specific NAbs following injection of rAd35f5 confirms that the Ad5 fiber protein on this virus was intact and immunogenic. Importantly, these fiber-specific NAb titers exceeded the fiber-specific NAb titers observed in humans in the developing world (Fig. 2*a*).

We then performed denaturing SDS-PAGE and Western blots to confirm that these purified IgG preparations reacted with a 110-kDa band corresponding to the hexon protein of the appropriate Ad serotype. As expected, IgG from mice that received rAd5 or rAd5f35p35 contained Ad5 hexon- but not Ad35 hexon-specific Abs (Fig. 4*b*). Similarly, IgG from mice that received rAd35 or rAd35f5 contained Ad35 hexon- but not Ad5 hexon-specific Abs. We also detected limited cross-reactivity (<20% by gel densitometry) to a 60- to 65-kDa protein, which likely represents the IIIa assembly protein (66 kDa), the penton protein (63 kDa), or both. We were not able to detect bands corresponding to the fiber proteins on these gels, because fiber-specific NAbs recognize primarily conformational determinants (9). Bands corresponding to the fiber proteins were detected using native SDS-PAGE and similar Western blots (data not shown).

We performed adoptive transfer studies using these purified IgG preparations containing high titers of Ad5-specific NAbs. Groups of naive recipient mice ($n$ = 4/group) received 500 $\mu$l of purified IgG before immunization with $10^8$ vp of rAd5-Env. To verify circulating NAb titers in these animals, serum was obtained after adoptive transfer and immediately before rAd5-Env vaccination. Serum NAbs in the recipient mice following adoptive transfer exhibited 4-fold lower titers compared with the titers present in the IgG preparations, as expected based on the dilutional effects of circulating volume (Fig. 4*c*).

We assessed Env-specific cellular and humoral immune responses following rAd5-Env vaccination using tetramer binding assays, IFN-$\gamma$ ELISPOT assays, and ELISAs. As depicted in Fig. 4*d*, adoptive transfer of IgG from mice that received rAd35 or rAd35f5 (●, ■) did not detectably suppress tetramer$^+$CD8$^+$ T lymphocyte responses. These data suggest that even high titers of Ad5 fiber-specific NAbs did not substantially suppress rAd5-Env

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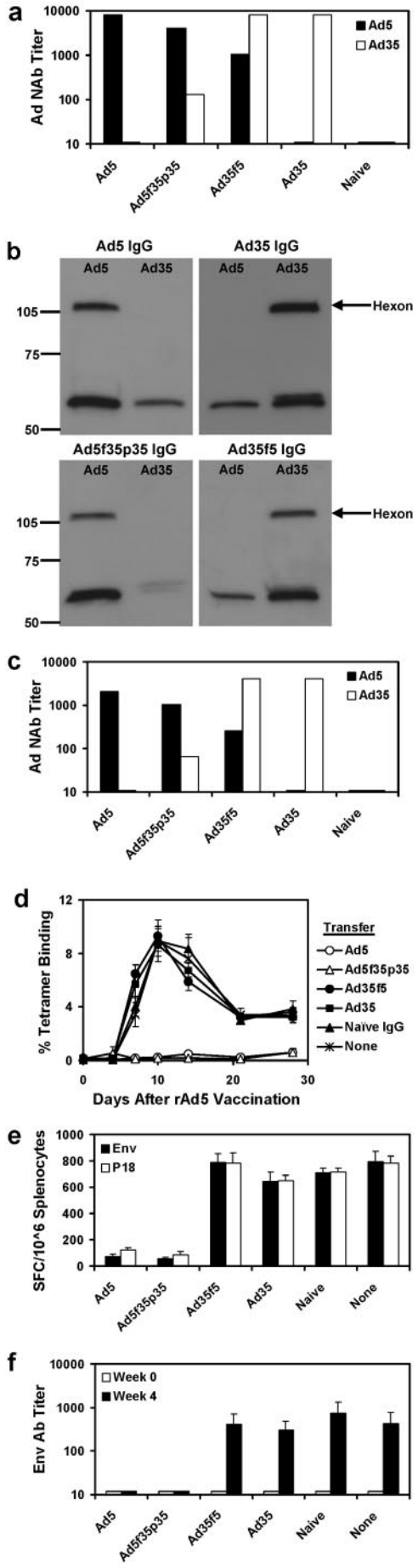


vaccine immunogenicity in this system. In contrast, adoptive transfer of IgG from mice that received rAd5 or rAd5f35p35 (○, △) completely abrogated peak and memory tetramer$^+$CD8$^+$ T lymphocyte responses, demonstrating the dramatic suppressive effects of Ad5 hexon-specific NAbs ($p < 0.001$ comparing mean tetramer responses among groups on day 10 and day 28 following immunization). Functional pooled peptide and epitope-specific ELISPOT assays (Fig. 4*e*) and Env-specific ELISAs (*f*) showed similar suppressive effects of these IgG preparations ($p < 0.001$). Thus, hexon-specific NAbs were substantially more effective than fiber-specific NAbs in abrogating rAd5 vaccine-elicited cellular and humoral immune responses.

To assess the generalizability of these results, we repeated this adoptive transfer study in a second experimental system. C57BL/6 mice were preimmunized twice with $10^{10}$ vp of rAd5, rAd5f35p35, rAd35f5, rAd35, or saline as above, and purified IgG was adoptively transferred to naive recipient mice before immunization with $10^8$ vp of rAd5-Gag. Ad5- and Ad35-specific NAb titers in these IgG preparations and in serum samples obtained from the mice before vaccination were comparable to those in the previous study (data not shown). Vaccine-elicited immune responses were assessed by D$^b$/AL11 tetramer binding assays and Gag-specific ELISAs. As shown in Fig. 5, adoptive transfer of IgG from mice that received rAd35f5 showed a slight and transient trend toward reduction of tetramer$^+$CD8$^+$ T lymphocyte responses on day 14 following immunization. Thus, it is possible that fiber-specific NAbs may have a secondary role in suppressing vaccine immunogenicity in certain settings. However, adoptive transfer of IgG from mice that received rAd5 or rAd5f35p35 resulted in significant and durable suppression of vaccine-elicited cellular ($p < 0.01$) and humoral ($p < 0.001$) immune responses. These studies confirm our previous findings that functionally significant Ad5-specific NAbs are directed primarily against the Ad5 hexon protein.

## Discussion

The high prevalence of pre-existing anti-Ad5 immunity in human populations may substantially limit the immunogenicity and clinical utility of rAd5 vector-based vaccines for HIV-1 and other pathogens. Our studies demonstrate >90% Ad5 seroprevalence in sub-Saharan Africa with median NAb titers >10-fold higher than those found in the United States. These data suggest that rAd5 vectors should be engineered to evade dominant Ad5-specific NAbs before their use as vaccine vectors in the developing world. To determine the principal targets of Ad5-specific NAbs, we exploited the lack of detectable serologic cross-reactivity between Ad5 and Ad35 (8). Virus neutralization studies using capsid chimeric rAd5/rAd35 vectors and serum samples from both humans

**FIGURE 4.** Adoptive transfer studies using high titers of Ad-specific NAbs. IgG was purified from pooled serum from BALB/c mice that received two injections of $10^{10}$ vp of rAd5, rAd5f35p35, rAd35f5, rAd35, or saline. *a*, The IgG preparations were assessed for NAbs to rAd5 and rAd35. *b*, Western blots were performed to confirm reactivity of these IgG preparations with the 110-kDa hexon band of the expected serotype. The IgG preparations were then adoptively transferred into naive BALB/c mice ($n = 4$/group) by tail vein injection before immunization with $10^8$ vp of rAd5-Env. *c*, Serum was obtained from these mice following adoptive transfer and immediately before vaccination and assessed for NAbs to rAd5 and rAd35. *d–f*, Vaccine-elicited immune responses following immunization were determined by D$^d$/P18 tetramer binding assays (*d*), Env pooled peptide and P18 epitope-specific ELISPOT assays (*e*), and Env-specific ELISAs (*f*). Controls included mice that received adoptive transfer of naive IgG purified from saline-injected mice (Naive) and mice that received no adoptive transfer (None).

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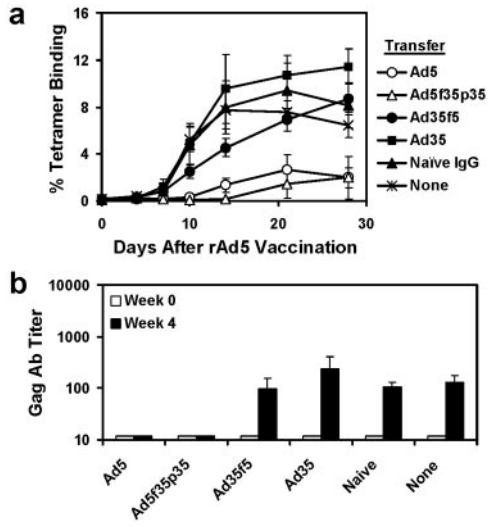


**FIGURE 5.** Adoptive transfer studies using high titers of Ad-specific NAbs in C57BL/6 mice. IgG was purified from pooled serum from C57BL/6 mice that received two injections of $10^{10}$ vp of rAd5, rAd5f35p35, rAd35f5, rAd35, or saline. These IgG preparations were adoptively transferred into naive C57BL/6 mice ($n = 4$/group) by tail vein injection before immunization with $10^8$ vp of rAd5-Gag. Vaccine-elicited immune responses following immunization were determined by $D^b$/AL11 tetramer binding assays (*a*) and Gag-specific ELISAs (*b*). Controls included mice that received adoptive transfer of naive IgG purified from saline-injected mice (Naive) and mice that received no adoptive transfer (None).

and mice demonstrated that Ad5-specific NAbs were directed primarily against the Ad5 hexon protein. Fiber-specific NAbs were detected at low frequencies in vitro but were substantially less efficient than hexon-specific NAbs at blunting rAd5 vaccine immunogenicity in vivo in adoptive transfer studies in mice.

The present studies extend prior reports that have demonstrated potent neutralizing activity of hexon-specific Abs in vitro (18, 19, 32). Previous studies have also shown that chimeric rAd5 vectors containing the Ad2, Ad6, or Ad12 hexon genes were able to evade a substantial fraction of anti-Ad5 immunity in rodents (20–22), although these studies did not differentiate between the contributions of hexon-specific humoral and cellular immunity. In contrast with these reports, a recent clinical study suggested that fiber- and penton-specific Abs were in fact substantially higher titer than hexon-specific Abs in humans using Western blot analyses (23). Another clinical study similarly reported that Ad5-specific Abs recognized primarily fiber and penton determinants and that these Abs exhibited synergistic neutralizing activity (24). In the present studies, we demonstrate that hexon-specific NAb titers were 4- to 10-fold higher than fiber-specific NAb titers in humans from both the United States and the developing world as well as in immunized mice. These data suggest that there is no major difference in the Ad5-specific NAb profiles in humans and rodents. We speculate that the differences between our results and these prior clinical studies may reflect the efficiency with which hexon-specific Abs neutralize viral particles. As a result, Western blots using purified proteins (23, 24) may underestimate the functional capacity of hexon-specific Abs to neutralize virus.

We used adoptive transfer studies to show that hexon-specific NAbs were substantially more potent than fiber-specific NAbs in blunting rAd5 vaccine immunogenicity in mice. Interestingly, the lack of substantial suppression of rAd5 vaccine immunogenicity by fiber-specific NAbs could not be fully explained by their lower titers compared with hexon-specific NAbs. In fact, mice that received two injections of rAd35f5 had clearly detectable Ad5-specific NAbs that were presumably directed against fiber (Fig. 4*a*). However, even these relatively high titers of fiber-specific NAbs exerted minimal effects on rAd5 vaccine immunogenicity. In contrast, low titers of hexon-specific NAbs were sufficient to suppress rAd5 immunogenicity (Fig. 3). These observations suggest that blunting rAd5 immunogenicity in vivo is a more complex process than neutralizing rAd5 infection of cells in vitro.

Previous studies have reported differences in the kinetics and mechanism of neutralization by hexon-, fiber-, and penton-specific NAbs. Hexon-specific NAbs function by rapid, single-hit kinetics, and are believed to inhibit the pH-dependent conformational changes of viral particles in endosomes before their entry into the cytosol (19). Importantly, hexon-specific NAbs have the ability to neutralize viral particles that are already attached to cells, which likely contribute to their efficient suppression of rAd5 vaccine immunogenicity in vivo. In contrast, fiber- and penton-specific NAbs have substantially slower kinetics of neutralizing free virus and are unable to neutralize viral particles following attachment to cells (19). Fiber-specific NAbs are thought to function by inducing aggregation of viral particles before cellular attachment, and penton-specific NAbs may inhibit viral uncoating (19, 23). These kinetic and mechanistic differences may contribute to the differences in efficiency among hexon-, fiber-, and penton-specific NAbs in suppressing rAd5 vaccine immunogenicity in vivo.

Regardless of the precise mechanism, our studies indicate that hexon-specific NAbs are substantially more efficient than fiber-specific NAbs in suppressing rAd5 vaccine immunogenicity. Consistent with this finding, we have previously observed that rAd35f5 vaccine vectors were unable to bypass anti-Ad5 immunity in mice (33). We were unable to evaluate directly the suppressive effects of penton-specific NAbs using our panel of capsid chimeric rAd5/rAd35 viruses. However, the comparable NAb titers to rAd5f35 and rAd5f35p35 in both humans and rAd5-immunized mice (Fig. 2) suggest that penton-specific NAbs represented at most a minor component of total Ad5-specific NAbs. Moreover, the comparable NAb titers to rAd5 and rAd5f35p35 in these samples suggested that the combination of fiber- and penton-specific NAbs also played a minor role, although we cannot rule out the possibility of low levels of synergistic fiber- and penton-specific NAbs (24).

We conclude that functionally significant Ad5-specific NAbs are directed primarily against the Ad5 hexon protein, although we cannot rule out the possibility that NAbs against other capsid components may also prove relevant in certain settings. Nevertheless, these findings could potentially be exploited in ongoing efforts to engineer Ad5 vectors to evade dominant Ad5-specific NAbs. For example, it may be possible to remove or mutate dominant NAb epitopes in the Ad5 hexon protein. In fact, exchanging complete hexon genes among viruses within Ad subfamily C (Ad2, Ad5, Ad6) has already been shown to result in chimeric viruses that partially evaded anti-Ad5 immunity (20, 22). However, chimeric Ad5 vectors containing hexon genes from other subfamily C Ads will not likely have practical utility, because all subfamily C Ads have a high seroprevalence in humans (13). Unfortunately, exchanging complete hexon genes among Ads from other subfamilies has been shown to be complicated by viral structural constraints (20–22). Future work could therefore explore the possibility that smaller or more defined hexon mutations may allow the construction of novel Ad5 vectors that may evade a substantial fraction of dominant hexon-specific NAbs. Such vectors may prove substantially more immunogenic than the current generation of rAd5 vaccines, particularly in individuals in the developing world with high levels of anti-Ad5 immunity.

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## Disclosures

The authors have no financial conflict of interest.

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