Adenovirus Type 4 and 7 Vaccination or Adenovirus Type 4 Respiratory Infection Elicits Minimal Cross-Reactive Antibody Responses to Nonhuman Adenovirus Vaccine Vectors

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Antivector immunity may limit the immunogenicity of adenovirus vector vaccines. We tested sera from individuals immunized with adenovirus type 4 and 7 (Ad4 and Ad7, respectively) vaccine or naturally infected with Ad4 for their ability to neutralize a panel of E1-deleted human and chimpanzee adenoviruses (ChAd). Small statistically significant increases in titers to ChAd63, ChAd3, human Ad35, and human Ad5 were observed. Neutralizing antibodies elicited by Ad4 infection or immunization results in a small amount of adenovirus cross-reactivity.

Adenoviruses (Ad) are promising candidates as vectors for infectious disease and cancer vaccines designed to elicit T-cell activation and cellular immune responses (1–3). However, preexisting immunity may limit their usefulness as vaccines, which has been described for recombinant human Ad type 5 (rAd5) (4–8) and was attributed to the presence of neutralizing antibodies (nAb). As a result, an alternative approach has been to utilize uncommon human or closely related animal adenovirus types as potential vectors (9). Chimpanzee adenovirus (ChAd) vectors demonstrated efficacy in a nonhuman primate model of Ebola virus infection and for hepatitis C and *Plasmodium falciparum* malaria (10, 11), and they appear to be unaffected by preexisting Ad5 immunity (N. Sullivan, unpublished observations). However, the concern that cross-reactive neutralizing activity may im-

pair the immunogenicity of these alternative vectors persists (12). Specifically, ChAd63 and Ad4 are classified within the species E serogroup of adenoviruses. Ad4 infection is fairly common in adult populations (34 to 45% adult seroprevalence) (9, 13, 14) and

TABLE 1 Pre- and postinfection or vaccination titers by adenovirus serotype

| Serotype and time point (no. of samples)[a]	Prevalence of titers ≥200 (% [95% CI])[b]	GMT (95% CI)[c]	Fold change in GMT	No. (%) of responders[d]	P[e]
Ad4					
Pre (50)	0 (0–7.1)	11.3 (10.7–12.0)			
Post (50)	70.0 (55.4–82.1)	578.0 (400.5–834.1)	51.2	50 (100)	<0.0001
Ad5					
Pre (31)	40.6 (23.7–59.4)	135.2 (54.3–336.4)			
Post (31)	46.9 (29.1–65.2)	250.4 (99.5–630.4)	1.9	5 (16.1)	0.0001
Ad26					
Pre (32)	6.3 (0.8–20.8)	15.3 (10.4–22.4)			
Post (32)	12.5 (3.5–29.0)	18.4 (11.1–30.4)	1.2	1 (3.1)	0.25
Ad35					
Pre (29)	0 (0–11.9)	12.1 (9.9–14.8)			
Post (29)	13.8 (3.9–31.7)	30.3 (15.8–58.2)	2.5	6 (20.7)	0.0005
ChAd3					
Pre (43)	27.9 (15.3–43.7)	62.0 (35.8–107.3)			
Post (43)	51.2 (35.4–66.7)	181.9 (96.7–342.3)	2.9	12 (27.9)	<0.0001
ChAd63					
Pre (38)	0 (0–9.3)	11.1 (10.9–11.2)			
Post (38)	7.9 (1.7–21.4)	21.4 (14.3–31.9)	1.93	4 (10.5)	0.0002

[a] Ad, adenovirus; Ch, chimpanzee.
[b] CI, confidence interval.
[c] Eleven was used as minimum for calculations based on negative cutoff value of 12. GMT, geometric mean titer.
[d] Defined as 4-fold increase in titer.
[e] P values calculated based on Wilcoxon matched-pairs test.
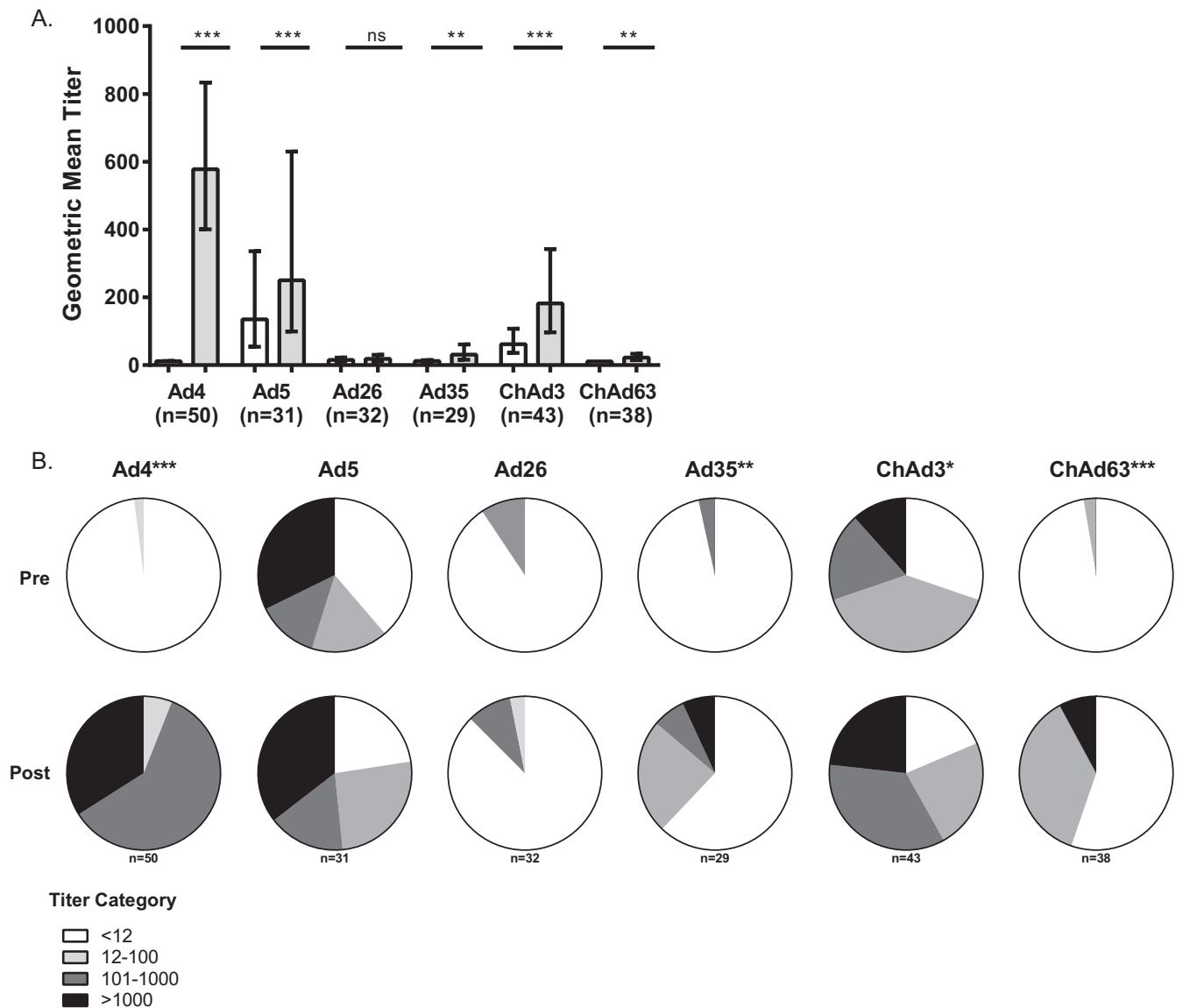
FIG 1 Vaccination or infection with Ad4 is associated with a small increase in neutralizing activity against common and rare human and primate adenoviruses (white bars represent pre-Ad exposure and gray bars post-Ad exposure titers). (A) Geometric mean titers (GMTs) and 95% confidence intervals (CI) for all subjects. The Kruskal-Wallis test was used to assess statistical significance. (B) Pie charts depicting frequency of pre-Ad exposure and post-Ad exposure titers by titer range. The Chi-square test was used to test statistical significance. There was insufficient sample to test sera against all isolates. *, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.0001$. ns, not significant.

is a common cause of acute febrile respiratory illness (AFRI) among military recruits (15, 16). Currently, all U.S. military recruits receive the licensed live oral types 4 and 7 adenovirus vaccine during basic training. To assess for potential antivector immunity, we tested sera from vaccinated and naturally infected subjects against a panel of E1-deleted adenoviruses that included Ad4, Ad5, Ad26, Ad35, ChAd3, and ChAd63.

We obtained fifty paired serum samples from a clinical trial of oral Ad4 and Ad7 vaccine ($n = 19$) in military recruits and in civilians (17) and from a prospective study of AFRI with Ad4 infection at a U.S. Army recruit training center ($n = 31$) (18). All samples were tested at the time of the initial studies and were negative for Ad4 nAb at baseline and either positive or negative for Ad7 nAb. Blood specimens were obtained prior to vaccination or the onset of illness and from 2 to 8 weeks afterward. All sera were

tested concurrently in a luciferase reporter gene virus neutralization assay as previously described (19). Briefly, A549 human lung carcinoma cells (or 2393/T17 cells for ChAd63) were plated at a density of 1×10^4 cells per well in 96-well plates and infected with E1-deleted replication-incompetent rAd-luciferase reporter constructs of different serotypes (Table 1) at a multiplicity of infection of 500, with 2-fold serial dilutions of serum in 200-μl reaction volumes. Following a 24-hour incubation, the 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. We calculated the frequencies of samples exhibiting cross-neutralization and geometric mean titers (GMTs) for pre- and postexposure samples. An Ad nAb titer of >200 was used to stratify the analyses in a phase IIb HIV vaccine trial (20) and was

considered potentially detrimental to use of the vector in this study. In addition, we defined a positive response as a ≥4-fold increase in the titer over baseline. We used nonparametric tests to compare GMTs and the Chi-square test for categorized titers (<12, 12 to 100, 101 to 1,000, and >1,000). The participating institutional review boards (IRBs) approved all the studies from which the samples were collected. The Walter Reed Army Institute of Research and National Institutes of Health IRBs approved this study.

The postinfection GMTs for Ad4 and other adenoviruses in the AFRI cohort ($n = 31$) were higher than the postvaccination cohort ($n = 19$) GMTs (for Ad4, 652.6 [95% confidence interval (CI), 412.1 to 1,033] versus 474.2 [95% CI, 246.5 to 912.2], respectively), but the differences were not statistically significant ($P = 0.41$ for Ad4). There were no statistically significant differences in the GMTs between the infection and vaccination groups for the other adenoviruses tested (data not shown); therefore, we combined data from both groups for our analyses. We observed small (2- to 3-fold) but statistically significant increases in the GMTs after vaccination or infection with Ad4 for all viruses tested except for Ad26 (Fig. 1A, Table 1). The GMTs observed were mostly of low magnitude (<200), except for ChAd3 (for which 51.2% of GMTs were ≥200 postinfection/vaccination). When the GMTs were grouped by category, statistically significant differences were observed for Ad4, Ad35, ChAd3, and ChAd63 (Fig. 1B). Evaluation of paired titers demonstrated boosting of cross-reactive responses and new responses (defined as a 4-fold rise in titer) (Table 1). Ad5 neutralization responses were not correlated with the responses to ChAd3, which is from the same serogroup C ($P = 0.34$ for preinfection titers and $P = 0.46$ for postinfection or vaccination titers, Spearman's correlation test). However, the responses to other Ad not tested might have been associated with this cross-reactivity (9). Overall, these results indicate a small but consistent cross-neutralization of several adenovirus serotypes after Ad4 immunization or infection.

Adenovirus type identification by neutralization has historically been associated with cross-reactivity, sometimes requiring further testing using agglutination or molecular methods (13, 21, 22). Adenovirus infection may also produce smaller, heterotypic antibody responses to other Ad types (23). We observed small nAb responses in the same serogroup (E, ChAd63) and in serogroups B (Ad35) and C (Ad5, ChAd3). The largest heterotypic response was to ChAd3 (28%). We did not detect any differences between vaccination and infection, suggesting that these results are likely attributable to Ad4 rather than to Ad7. Previous studies have demonstrated decreased T-cell responses to Ad5 vector vaccines with preexisting Ad5 neutralization (8, 20, 24). In one study, preimmunization Ad5 titers of <12 were associated with a 3.29-times-higher enzyme-linked immunosorbent spot assay (ELISpot) response that that of seropositive subjects (25). The high prevalence of nAb to ChAd3 (27.9%) and the increase in GMTs following vaccination or infection suggest that this may not be a good candidate vector in this study population. One caveat to our study is that vaccination with a replication-deficient Ad vector may induce less cross-reactivity than we observed with a live replication-competent vaccine.

In summary, reporter gene-based neutralization assays can be used to quickly detect potential antivector immunity when there is concern for cross-reactivity. Our results are consistent with previous prevalence studies of antibodies to human and chimpanzee adenoviruses (4, 12, 26) and demonstrate that infection or vaccination with a more common adenovirus serotype may generate or boost cross-reactive antibodies. Further study is needed to establish the ability, if any, of these levels of neutralization activity to impair vector immunity.

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