Characterization of Humoral and Cellular Immune Responses Elicited by a Recombinant Adenovirus Serotype 26 HIV-1 Env Vaccine in Healthy Adults (IPCAVD 001)

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(See the major article by Baden et al, on pages 240–7.)

Background. Adenovirus serotype 26 (Ad26) has been developed as a novel candidate vaccine vector for human immunodeficiency virus type 1 (HIV-1) and other pathogens. The primary safety and immunogenicity data from the Integrated Preclinical/Clinical AIDS Vaccine Development Program (IPCAVD) 001 trial, the first-in-human evaluation of a prototype Ad26 vector-based vaccine expressing clade A HIV-1 Env (Ad26.ENVA.01), are reported concurrently with this article. Here, we characterize in greater detail the humoral and cellular immune responses elicited by Ad26.ENVA.01 in humans.

Methods. Samples from the IPCAVD 001 trial were used for humoral and cellular immunogenicity assays.

Results. We observed a dose-dependent expansion of the magnitude, breadth, and epitopic diversity of Env-specific binding antibody responses elicited by this vaccine. Antibody-dependent cell-mediated phagocytosis, virus inhibition, and degranulation functional activity were also observed. Env-specific cellular immune responses induced by the vaccine included multiple CD8+ and CD4+ T-lymphocyte memory subpopulations and cytokine secretion phenotypes, although cellular immune breadth was limited. Baseline vector-specific T-lymphocyte responses were common but did not impair Env-specific immune responses in this study.

Conclusion. Ad26.ENVA.01 elicited a broad diversity of humoral and cellular immune responses in humans. These data support the further clinical development of Ad26 as a candidate vaccine vector.

Clinical Trials Registration. NCT00618605.

Keywords. HIV-1; Vaccine; Adenovirus 26; Immunogenicity.
METHODS

immunologic pro
strate the broad diversity of immune responses and the unique assays, and cytokine multiplex bead arrays. These data demon-
mapping, multiparameter intracellular cytokine staining (ICS) assays. Cellular immune responses were assessed by epitope body responses and by functional nonneutralizing antibody.

In an accompanying manuscript [20], we describe the primary safety and immunogenicity results of the Integrated Preclinical/Clinical AIDS Vaccine Development Program (IPCAVD) 001 trial, a randomized, double-blinded, placebo-controlled, first-in-human phase 1 clinical trial of a prototype Ad26 vector–based vaccine expressing clade A HIV-1 Env (Ad26.ENVA.01). Ad26.ENVA.01 proved safe and immunogenic in healthy, Ad26-seronegative, HIV-1–uninfected subjects, and Env-specific humoral and cellular immune responses were assessed by enzyme-linked immunosorbent assays (ELISAs) and interferon γ (IFN-γ) enzyme-linked immunosorbent spot (ELISPOT) assays [20].

Here, we report a detailed characterization of humoral and cellular immune responses elicited by Ad26.ENVA.01 in humans in the IPCAVD 001 trial. Humoral immune responses were assessed by linear peptide arrays to evaluate magnitude, breadth, and epitopic diversity of Env-specific binding antibody responses and by functional nonneutralizing antibody assays. Cellular immune responses were assessed by epitope mapping, multiparameter intracellular cytokine staining (ICS) assays, and cytokine multiplex bead arrays. These data demonstrate the broad diversity of immune responses and the unique immunologic profile induced by this vaccine.

RESULTS

Env-Specific Humoral Immune Responses

As described in the primary manuscript associated with the IPCAVD 001 trial [20], we evaluated the safety and immunogenicity of a novel Ad26.ENVA.01 vaccine in humans at doses of 10^6 vp, 10^{10} vp, and 10^{11} vp (groups 1–3) administered at weeks 0, 4, and 24, as well as at doses of 5 \times 10^{10} vp and 10^{10} vp (groups 4 and 5) administered at weeks 0 and 24. Vector concentration was determined by spectrophotometry. ELISAs revealed that Ad26.ENVA.01 induced consistent and boostable Env-specific binding antibodies in all groups, although HIV-1–specific neutralizing antibody responses were not detected [20]. Here, we extend these humoral immune data by assessing the magnitude, breadth, and epitopic diversity of Env-specific binding antibody responses, as well as functional nonneutralizing antibody responses.

We first used peptide microarrays (JPT Peptide Technologies) to evaluate the capacity of antibodies in serum at peak immunity at week 28 (4 weeks following the final immunization) to bind linear peptides spanning multiple HIV-1 Env sequences from clades M, A, B, C, D, CRF02, and CRF01. This assay detects binding antibody responses against linear epitopes but not conformational epitopes. As shown in Figure 1, we observed robust cross-clade binding antibody responses to peptides in the V3 loop region in all groups. The largest number of peptide responses was observed in subjects who received the 10^{11} vp dose followed by the 5 \times 10^{10} vp dose, showing that higher vaccine doses resulted in not only higher...
magnitude responses but also increased breadth and epitopic diversity. For example, responses against the V2 loop [29] were observed with vaccine doses $>10^{10}$ vp. No responses were detected by these assays in the placebo recipients. Broad binding antibody responses against multiple clades were observed at the highest dose.

We next assessed functional nonneutralizing antibody responses in individuals who received vaccine doses of $10^9$ vp, $10^{10}$ vp, and $10^{11}$ vp. As shown in Figure 2A, induction of antibody-dependent cellular phagocytosis (ADCP) was evident in some vaccinees at week 8 and was observed in 90% of vaccinees in the $10^{10}$ and $10^{11}$ vp groups at peak immunity at week 28. These ADCP responses waned over time but were augmented following a homologous boost immunization. Antibody-dependent cell-mediated virus inhibition (ADCVI) and degranulation responses were also observed in a subset of vaccinees. No responses were detected in placebo recipients (data not shown). As depicted in Figure 2B, serum antibody responses often included multiple functions, with triple function (ADCP/ADCVI/degranulation) responses observed most frequently in subjects who received the higher vaccine doses and following the boost immunization. These data demonstrate that multiple nonneutralizing functional antibody responses were induced by Ad26.ENVA.01, although the protective capacity of these antibody responses remains to be determined.

We also assessed the isotypes of the antibodies induced by this vaccine. As shown in Figure 2C, Env-specific binding antibodies at week 8 and week 28 were $>90$% IgG1, with minor contributions of IgG3. Serum Ig A responses were not detected (data not shown). IgG1 and IgG3 antibody isotypes readily

Figure 1. Env-specific binding antibody profiles by peptide arrays. Sera from baseline and week 28 were assessed using peptide arrays (JPT Peptide Technologies) to assess linear antibody responses to peptides spanning Env from clades M, A, B, C, D, CRF02, and CRF01. Mean signal intensity (week 28 with baselines subtracted) from all subjects in each group is depicted. Black asterisks denote peptides with significantly elevated mean signal intensity as compared with placebos ($P<.05$, by Wilcoxon Mann–Whitney U tests), and red asterisks denote trends ($P<.10$).
bind particular Fc receptors, suggesting that antibodies induced by Ad26.ENVA.01 may be efficient at recruiting innate immune effector functions. Interestingly, the IgG1 and IgG3 profile observed in RV144 parallels the antibody profile observed in this study, whereas antibody responses in VAX003 were characterized by enhanced levels of IgG2 and IgG4 (G. Alter et al, unpublished data).

**Figure 2.** Env-specific functional nonneutralizing antibody binding responses and antibody isotypes. A, Serum from multiple time points from subjects who received $10^9$ viral particles (vp), $10^{10}$ vp, and $10^{11}$ vp of the vaccine were assessed for antibody-dependent cellular phagocytosis (ADCP), cellular inhibition (ADCVI), and macrophage inflammatory protein 1β degranulation. Bars represent mean responses. B, Percentage of vaccinees in these groups at week 8 and week 28 that exhibit multiple antibody functions (ADCP/ADCVI/degranulation). C, Immunoglobulin G (IgG) isotypes of Env gp140-, gp120-, and gp41-specific binding antibodies from week 8 and week 28 from subjects who received $10^9$ vp, $10^{10}$ vp, and $10^{11}$ vp of the vaccine are shown in pie charts. Mean proportional IgG1, IgG2, IgG3, and IgG4 responses are shown for each group.

**Env-Specific Cellular Immune Responses**

In the primary manuscript associated with the IPCAVD 001 trial, we report that Ad26.ENVA.01 elicited IFN-γ ELISPOT assay responses in all groups, with no clear dose response over the range of doses studied and with durability of responses for 52 weeks [20]. Here, we extend these data with additional studies to define the cellular immune breadth,
T-lymphocyte subpopulations, and cytokine secretion phenotypes elicited by this vaccine. For these studies, we selected group 4 for detailed study because the $5 \times 10^{10}$ vp dose represents the vector dose selected for further clinical development.

Epitope mapping studies revealed the induction of a median of 1 epitope (range, 0–3 epitopes) per individual (data not shown), suggesting limited cellular immune breadth to this prototype clade A Env immunogen. Multiparameter ICS assays demonstrated that the vaccine elicited both central memory (CD27+CD45RO+) and effector memory (CD27–CD45RO+) CD8+ and CD4+ T-lymphocyte responses, as shown in Figure 3A. Positive total and central memory CD8+ ICS responses were detected in 67% and 100% of subjects, respectively, at week 4. CD4+ T-lymphocyte responses were approximately 4-fold lower than CD8+ T-lymphocyte responses. Moreover, Env-specific CD8+ and CD4+ T lymphocytes at peak immunity at week 28 secreted multiple cytokines, including IFN-γ, interleukin 2 (IL-2), and tumor necrosis factor-α (TNF-α), and upregulated CD40L (CD154), as depicted in Figure 3B. A broad range of response magnitudes was observed (0.03%–2.04% of CD8+ T lymphocytes and 0.01%–0.07% of CD4+ T lymphocytes for IFN-γ secretion).

Additional cytokine secretion phenotype data were generated by stimulation of PBMCs from week 28 with pooled clade A Env peptides and analysis of cytokines in culture supernatants. As shown in Figure 4A, multiple cytokines were induced, in particular granulocyte-macrophage colony-stimulating factor, IFN-γ, IL-2, interleukin 10, interleukin 13, macrophage inflammatory protein 1β, and TNF-α. Taken together, these data demonstrate that Ad26.ENVA.01 elicited limited cellular immune breadth but induced a diversity of T-lymphocyte subpopulations and cytokine secretion phenotypes.

We also extended the cellular immune analysis to evaluate memory B-lymphocyte responses in PBMCs obtained during week 28 from this group, using a B-cell ELISPOT assay. As depicted in Figure 4B, we observed that 100% of vaccinees demonstrated B-lymphocyte responses to the clade B BaL and consensus ConS gp140 antigens, with 0.1%–1.7% of B lymphocytes responding to BaL and 0.4%–8.3% of B lymphocytes responding to ConS. These data are consistent with the binding antibody data (Figure 1) and show that Ad26.ENVA.01 induced robust cross-clade, Env-specific memory B-lymphocyte responses.

**Vector-Specific Cellular Immune Responses**

All individuals in the IPCAVD 001 trial were selected to be Ad26 seronegative at study entry, and thus this study does not address whether baseline Ad26 neutralizing antibodies may
Humoral and Cellular Immunogenicity of Ad26.ENV A.01

**Figure 4.** Env-specific cytokine secretion and B-lymphocyte enzyme-linked immuno spot (ELISPOT) assay responses. A, Peripheral blood mononuclear cells (PBMCs) from week 28 from subjects who received $5 \times 10^{10}$ viral particles of the vaccine were assessed for secretion of multiple cytokines following stimulation with pooled clade A Env peptides by cytokine multiplex bead arrays. Concentrations of each cytokine are shown. Bars reflect mean responses. GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN-γ, interferon γ; IL-2, interleukin 2; IL-3, interleukin 3; IL-4, interleukin 4; IL-5, interleukin 5; IL-9, interleukin 9; IL-10, interleukin 10; IL-13, interleukin 13; MIP-1β, macrophage inflammatory protein 1β; TNF-α, tumor necrosis factor α; TNF-β, tumor necrosis factor β. B, PBMCs from week 28 from subjects who received $5 \times 10^{10}$ vp of the vaccine were assessed for memory B-lymphocyte responses following stimulation with purified Bal or ConS gp140 in B-lymphocyte ELISPOT assays. Bars reflect mean responses. Average placebo assay backgrounds are shown as dotted lines.

impact vaccine immunogenicity. However, as shown in Figure 5A, we observed that nearly all (>95%) subjects who received the $10^9$ vp, $10^{10}$ vp, and $10^{11}$ vp doses of the vaccine had detectable Ad26-specific cellular immune responses at baseline, as measured by vector-specific ELISPOT assays that used either purified Ad26 virus or overlapping Ad26 hexon peptides for stimulation. These responses likely reflect Ad-specific T lymphocytes that are cross-reactive across multiple Ad serotypes, consistent with previous reports from our laboratories and others [29, 31–33]. As shown in Figure 5B, baseline Ad26-specific cellular immune responses at week 0 were not correlated with Env-specific ELISA or ELISPOT assay responses following vaccination at week 8 ($R^2 = 0.00–0.01$; $P = .69–.87$). Similarly, baseline Ad26-specific cellular immune responses were not correlated with Env-specific ELISA or ELISPOT assay responses at week 28 (data not shown). These data show that baseline Ad26-specific T-lymphocyte responses did not have a detectable impact on vaccine immunogenicity in this study.

**DISCUSSION**

In this article, we describe detailed humoral and cellular immune responses elicited by Ad26.ENV A.01 in the IPCAVD 001 trial, the first-in-human evaluation of an Ad26 vaccine vector. We used state-of-the-art immunologic assays to complement the primary immunogenicity data, which are reported in an accompanying manuscript [20]. The magnitude, breadth, and epitopic diversity of binding antibody responses appeared to be dose dependent, and functional nonneutralizing antibody responses were observed. The vaccine elicited both B- and T-lymphocyte responses, multiple T-lymphocyte subpopulations and cytokine secretion phenotypes were induced. These data demonstrate that Ad26.ENV A.01 elicited a broad diversity of humoral and cellular immune responses that help define the unique immunologic profiles elicited by this vaccine vector.

Ad26 is biologically substantially different from the well-characterized Ad5 vector by lower titers of vector-specific neutralizing antibodies worldwide [12], the use of CD46 rather than the coxsackievirus and Ad receptor (CAR) as its primary cellular receptor [11], and the induction of different immune phenotypes in preclinical studies [13, 16]. Moreover, Ad26 vectors combined with either MVA vectors or Ad35 vectors afforded partial protection against both acquisition of infection and virologic control following heterologous SIVmac251 challenges in rhesus monkeys [19]. Ad vectors from other species, such as chimpanzees, are also being explored in clinical trials [34–36]. Moreover, in the present study, we evaluated Ad26 vectors expressing HIV-1 Env, which represents an important antigen for protection [19, 30]. Thus, Ad26.ENV A.01 has key differences from the Ad5-Gag/Pol/Nef vaccine that was used in the Step study [3].

The humoral immune responses elicited by Ad26.ENV A.01 demonstrated broad and potent binding antibody responses against multiple clades (Figure 1), as well as nonneutralizing functional activity (Figure 2). The lack of neutralizing activity induced by Ad26.ENV A.01, however, suggests that the use of improved or engineered Env antigen sequences and/or more potent heterologous prime-boost regimens may be desirable for future studies. Moreover, the induction of V2-specific antibody responses with vaccine doses of $>10^{10}$ vp (Figure 1) may be relevant in light of the RV144 correlates analysis that raised the hypothesis that V2-specific antibodies elicited by the ALVAC/gp120 vaccine may have reduced HIV-1 acquisition risk [30].

Ad26.ENV A.01 induced both CD8+ and CD4+ T-lymphocyte responses with a diversity of central and effector memory subpopulations and cytokine secretion phenotypes (Figures 3–4).
Cellular immune breadth, however, remained limited. Future studies are therefore planned to evaluate the immunogenicity of Ad26 vectors expressing bioinformatically optimized mosaic HIV-1 Gag/Pol/Env immunogens [9, 37], which expanded CD8+ and CD4+ T-lymphocyte breadth without compromising Env-specific antibody responses in rhesus monkeys [38]. A limitation of the present studies is that we were only able to assess peripheral immune responses in the IPCAVD 001 trial. Preclinical studies in both mice and rhesus monkeys have shown that Ad26 vectors delivered by the intramuscular route can also elicit robust mucosal immune responses [39–41]. Future studies are therefore warranted to assess antigen- and vector-specific mucosal immune responses and mucosal inflammatory responses elicited by Ad26 vectors in humans.

A previous study reported that baseline Ad5-specific T-lymphocyte responses inhibited the induction of antigen-specific cellular immune responses by Ad5-gag/pol/nef vaccine vectors in humans [31]. In contrast to these data, we did not observe an impact of baseline Ad26-specific T-lymphocyte responses on the subsequent development of Env-specific humoral or cellular immune responses in this study (Figure 5). We suspect that these differences may relate to biological differences between these Ad serotypes, differences in the characteristics of vector-specific cellular immune responses, and technical differences in the assays that were used. We cannot exclude the possibility that we may have missed a small effect, given the limited sample size of the present study.

In summary, we have shown that Ad26.ENVA.01 elicits a broad diversity of humoral and cellular immune responses in humans. However, the immune correlates of protection against HIV-1 infection in humans are not yet fully defined. Nevertheless, these data characterize the detailed immunologic profiles elicited

Figure 5. Adenovirus serotype 26 (Ad26)–specific T-lymphocyte responses by enzyme-linked immuno spot (ELISPOT) assays. A, Baseline Ad26-specific cellular immune responses were assessed by ELISPOT assays following stimulation with purified Ad26 virus or pooled Ad26 hexon peptides. B, Lack of correlation between baseline Ad26-specific cellular immune responses at week 0 and Env-specific humoral ELISA (top) and cellular ELISPOT assay (bottom) responses at week 8 from subjects who received 10⁹ viral particles (vp), 10¹⁰ vp, and 10¹¹ vp of the vaccine (Spearman rank-correlation tests).
by a prototype Ad26 vector in humans and suggest that further clinical development of Ad26 vaccine vectors is warranted.

Supplementary Data

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Potential conflicts of interest. M. G. P. M. W., and J. G. are employees of Crucell. Patents for the Ad26 vector are held in part by Crucell and BIDMC. No licensing agreements, royalties, or income is associated with these patents. All other authors report no potential conflicts.

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References