DNA gag/Adenovirus Type 5 (Ad5) gag and Ad5 gag/Ad5 gag Vaccines Induce Distinct T-Cell Response Profiles

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Received 19 March 2008/Accepted 28 May 2008

Results from Merck’s phase II adenovirus type 5 (Ad5) gag/pol/nef test-of-concept trial showed that the vaccine lacked efficacy against human immunodeficiency virus (HIV) infection in a high-risk population. Among the many questions to be explored following this outcome are whether (i) the Ad5 vaccine induced the quality of T-cell responses necessary for efficacy and (ii) the lack of efficacy in the Ad5 vaccine can be generalized to other vector approaches intended to induce HIV type 1 (HIV-1)-specific T-cell responses. Here we present a comprehensive evaluation of the T-cell response profiles from cohorts of clinical trial subjects who received the HIV CAM-1 gag insert delivered by either a regimen with DNA priming followed by Ad5 boosting (n = 50) or a homologous Ad5/Ad5 prime-boost regimen (n = 70). The samples were tested using a statistically qualified nine-color intracellular cytokine staining assay measuring interleukin-2 (IL-2), tumor necrosis factor alpha, macrophage inflammatory protein 1B, and gamma interferon production and expression of CD107a.

Both vaccine regimens induced CD4+ and CD8+ HIV gag-specific T-cell responses which variably expressed several intracellular markers. Several trends were observed in which the frequencies of HIV-1-specific CD4+ T cells and IL-2 production from antigen-specific CD8+ T cells in the DNA/Ad5 cohort were more pronounced than in the Ad5/Ad5 cohort. Implications of these results for future vaccine development will be discussed.

As the global human immunodeficiency virus type 1 (HIV-1) epidemic continues to expand, the development of a prophylactic vaccine against the virus remains a critical goal. A large segment of the current HIV-1 vaccine effort has included vaccines designed to induce cell-mediated immune responses due to the volume of data supporting the role of CD8+ T cells in the control of viral infection. Cytotoxic (CD8+) T cells have been demonstrated to directly kill HIV-1-infected CD4+ T cells (25) and have been associated with the control of viremia during primary HIV infection (6, 18). CD4+ T cells, which are also essential in the control of viral infections, exert antiviral responses including the production of cytokines that mediate effector functions (13, 28, 30) and provide help to support functional CD8+ T-cell responses (1, 22, 29).

Among the most advanced clinical HIV-1 vaccine candidates are (i) Merck’s replication-defective adenovirus type 5 (Ad5) viruses expressing clade B HIV-1 gag, pol, and nef and (ii) the National Institutes of Health’s vaccine which involves a heterologous DNA priming-Ad5 boosting regimen; the latter vaccine involves three env genes, one from each of the major clades (A, B, and C), plus clade B gag, pol, and nef. While the NIH vaccine included env genes, no neutralizing antibody activities were detected in human vaccine recipients (8); hence, it is likely the major contribution of the env genes will be to increase the breadth of anti-HIV T-cell responses. In preclinical studies, both approaches had shown partial efficacy in some simian immunodeficiency virus challenge model systems (7, 20, 26). In phase I trials, both vaccine approaches were shown to be well-tolerated and highly immunogenic (8, 9) (unpublished data).

In December 2004, the Ad5 gag/pol/nef vaccine entered a phase II test-of-concept study in 3,000 seronegative high-risk subjects. The study was designed to determine if the vaccine was able to reduce infection rates and/or reduce the set point viremia. On 18 September 2007, the data safety monitoring board (DSMB) recommended discontinuation of study vaccinations because of lack of vaccine efficacy (data from the trial can be viewed at http://www.hvtn.org/science/1107.html). In addition, more HIV-1 infections were found in the treatment group over the placebo cohort control. It is unclear if the lack of efficacy of the Ad5 gag/pol/nef vaccine is specific to the candidate or the concept itself of a vaccine-induced T-cell-based immunity. And in the case of the former, how might a DNA prime-Ad5 boosting strategy perform in a clinical efficacy trial?

To address this important question, it is essential to characterize the difference not only in magnitude of the immune responses but also in the quality of the induced T-cell responses between the two vaccine approaches. One valuable platform for this type of analysis is multicolor flow cytometry, which enables the concurrent examination of multiple T-cell functions (such as interleukin-2 [IL-2], tumor necrosis factor alpha [TNF-α], macrophage inflammatory protein 1B [MIP-1B], and gamma interferon [IFN-γ] production and expression of CD107a) on the single-cell level. The potential advantages of multifunctional T-cell responses have recently been demonstrated in a number of disease models.
27), including HIV (4, 16). Of particular interest are the studies which demonstrate that HIV controllers maintain more multifunctional CD8+ (4) and CD4+ (16) T cells than HIV noncontrollers. Therefore, the functional profiles of the T-cell response as measured by multicolor flow methodologies may be an important measurement in a vaccine trial setting, as this may correlate with a type of protective immune response.

We developed a nine-color flow cytometric assay which concurrently measures IL-2, TNF-α, MIP-1β, and IFN-γ production as well as the expression of CD107a. The assay was rigorously qualified to determine the appropriate positivity criteria, the associated false-positive rates, and the repeatability of positive responses. Here we tested 50 DNA gag/Ad5 gag-vaccinated and 70 Ad5gag/Ad5gag-vaccinated subjects for the purpose of characterizing the antigen-specific T-cell functional responses to the HIV CAM-1 gag immunogen as delivered by two different vaccine modalities. We show that both vaccine regimens induced multifunctional gag-specific CD4+ and CD8+ T-cell responses. Analyses were also extended to a cohort of the Ad5/Ad5 group which received a trivalent vaccine (Ad5 gag/pol/nef). The results demonstrate some important distinctions between the DNA/Ad5 and Ad5/Ad5 profiles, including more CD4+ responses and IL-2 expression from the DNA/Ad5 group.

![FIG. 1. Two-dimensional positivity criteria with HIV gag peptides. The HIV gag multi-ICS responses from 10 HIV-seronegative subjects are displayed for the CD8+ (A) and CD4+ (B) T-cell subsets. Each subject yielded 31 results (one result for each functional subset) for both CD4+ and CD8+ T cells, and each subject was tested twice, yielding 620 data points for each graph. Responses are shown in a two-dimensional region-of-positivity model, in which the natural logs of the peptide responses “P” are plotted against the natural logs of the “mock” nonstimulated responses, “M.” The region of positivity is defined by peptide events of ≥P₀ and a peptide/mock ratio of ≥R₀, for which we defined P₀ = 90 (vertical line) and R₀ = 3 (dashed horizontal line). Symbols in the “positive region” represent false positives in these samples.]

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a Abbreviations: v.p., virus particles; SFC, spot-forming cells; geomean, geometric mean; alum., aluminum.
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FIG. 2. Nine-color ICS gating strategy. The gating strategy for the enumeration of multifunctional CD8$^+$ and CD4$^+$ T-cell responses in the nine-color ICS assay is displayed. The data shown are a representative HIV gag-specific response from a DNA/Ad5 vaccine recipient. The gates were set based on the negative control (mock) samples and were set as synchronized gates within each sample. See Materials and Methods for additional details on the procedure.

MATERIALS AND METHODS

Study participants and vaccine information. Samples were collected from healthy HIV-negative volunteers, from HIV-positive subjects, and from HIV-negative experimental vaccine recipients. Collectively, the vaccinated subjects received one of the following vaccine candidates as administered in randomized phase I clinical trials in the United States: (i) DNA expressing clade B CAM-1 gag, JRFL nef, and IIIB pol; pol-1 is the NH2-terminal half, and pol-2 is the COOH-terminal half, and pol-3 is the open reading frame of Brefeldin A (Sigma-Aldrich), 100 μg/ml anti-CD49d (340976; BD Biosciences). Each peptide pool (15-mer) was added to achieve a final concentration of approximately 4.5 μg/ml. Peptide-free dimethyl sulfoxide (DMSO) diluent matching the DMSO concentration in the peptide solution served as a nonantigen control (“mock”) for each sample. Following a 5- to 6-h incubation period, 20 μl of 20 mM EDTA was added to each well. Cells were washed with fluorescence activated cell sorter buffer (phosphate-buffered saline plus 1% fetal bovine serum plus 0.01% sodium azide) and incubated for 30 min with the appropriately titrated volume of CD3-phycocerythrin (PE)-Cy5 (UCHT-1; BD Biosciences), CD4-allophycocyanin-Cy7 (RPA-T4; BD Biosciences), CD8–peridinin chlorophyll protein-Cy5.5 (SK-1; BD Biosciences), and violet amine reactive viability dye (L34955; Invitrogen) (19). Cells were washed twice with fluorescence-activated cell sorter buffer and permeabilized with 200 μl of Cytofix/Cytoperm solution (BD Biosciences) for 20 min at room temperature. Cells were washed twice with Perm/Wash buffer (BD Biosciences) and stained with the appropriately titrated volume of IL-2–allophycocyanin (534.111; BD Biosciences), TNF–PE-Cy7 (MAb11; BD Biosciences), IFN–fluorescein isothiocyanate (1-D1K; MabTech USA, Cincinnati, OH), MIP-1β–PE (D21-1351; BD Biosciences), and streptavidin-PE-Texas red (551487; BD Biosciences) for 60 min. The samples were then washed four times with BD Perm/Wash buffer and fixed with 1% formaldehyde. Plates were placed at 4°C overnight, and approximately 300,000 total events per sample were acquired the following day on an LSRII instrument (BD Biosciences).

Flow cytometric data analysis. A standardized FlowJo (Tree Star Inc., Ashland, OR) analysis template was constructed and used by trained assay operators. A synchronized group feature was used, and only one sample at a time was analyzed within the template. Therefore, all gate adjustments were identical for the mock result and all antigen results within a given sample. This template included a gate to define the lymphocyte population on a forward scatter versus side scatter plot and a CD3 versus amine-reactive dye dot plot with a gate on the CD3$^+$ viable population. A CD8 versus CD4 dot plot (gated on viable CD3$^+$ lymphocytes) was displayed with gates drawn around the CD8$^+$ and CD4$^+$ single-positive populations. For each T-cell subset, CD4$^+$ and CD8$^+$, five dot plots were displayed as side scatter versus CD107a, IFN-γ, MIP-1β, IL-2, or TNF-α. A gate on each of these graphs was set to include positive cells (see Fig. 2). FlowJo software’s Boolean gate feature was utilized in the template to create all possible positive and negative combinations of these functional markers. Excluding the populations that were negative for all markers, this analysis yielded...
62 results per test well, hereafter referred to as subsets. These results were exported from FlowJo into a spreadsheet and normalized to events per 1 million lymphocytes for the reported final results. These normalized values are hereafter referred to as the responses. The data analysis program Simplified Presentation of Incredibly Complex Evaluations (version 4.1.6; provided by M. Roederer, Vaccine Research Center, NIAID, NIH) was used to generate the graphical representation in Fig. 5.

Statistical analysis. (i) Hypothesis testing of the overall CD4+ or CD8+ T-cell profiles. The hypothesis that the cohorts had similar T-cell response profiles across the 31 functional combinations was tested using a likelihood ratio test. The 95th and 99th percentiles of this statistic for the null hypothesis that the proportion responding positively for each of the T-cell combinations was not different between groups were computed by simulating 10,000 values of the likelihood function under the assumption that each of the subsets for each of the groups came from a Bernoulli distribution.

(ii) Statistical analysis to compare the number of positive individuals for T-cell subsets. The null hypothesis that the proportion of individuals responding positively in a T-cell subset is the same as the proportion of individuals in the comparative cohort tested. The distribution was simulated, and the upper and lower 5th and 95th and 1st and 99th percentiles were established.

RESULTS

Nine-color ICS assay qualification. Prior to initiating testing of clinical trial specimens, we set out to define the parameters of the assay in order to support proper data interpretation. To establish a criterion for positivity, responses by 10 HIV-negative, nonvaccinated donors following stimulation with DMSO, gag, nef, pol-1, or pol-2 were measured twice to characterize the patterns of negative responses in the assay. It was observed that the response to the peptide pools and the response to the DMSO control were proportional and that different subjects, as well as distinct functional subsets, may have inherently different levels of background. Therefore, we evaluated the joint distribution of the measured DMSO result and the peptide antigen-stimulated result in a two-dimensional region of positivity. The limits of antigen counts and antigen/mock ratios were varied until they excluded background noise levels, and a limited number of results were in the region of positivity. When the antigen count was set to 90 and the DMSO ratio was set to 3, a limited number of results fell in the defined positive region of the graph (Fig. 1). Graphs from the nef, pol-1, and pol-2 data were very similar (data not shown). Therefore, a two-dimensional positivity cutoff of ≥90 antigen-specific events per million lymphocytes and ≥3-fold over background was established. Forty additional HIV-negative nonvaccinated subjects were tested to gather more data on assay false-positive (FP) rates using the established positivity criterion. Most of the resulting FP rates were near 1%, and the upper 95% limit was ≤5% for all of the subsets. Viewing the FP rates on a subset basis also demonstrated that none of the T-cell subsets had significantly elevated FP rates using these criteria, thereby giving us confidence in the ability to use a single positivity criterion for all of the subsets. Lastly, we calculated the assay repeatability by estimating the percent relative standard deviation (RSD) of the assay. Ten HIV-infected subjects that were known HIV gag peptide responders were chosen and tested with DMSO and the gag peptide pool in four separate multicolor ICS assay runs. This analysis yielded 56 sets of four assay results that were included in the calculation to estimate the percent RSD of the assay to be approximately 50%. No significant difference in percent RSD was observed when comparing results from the CD4+ and CD8+ subsets. These results indicate that this assay is highly repeatable and well-qualified for the purpose of testing clinical trial specimens with a high level of confidence in the data output.

Multifunctional CD8+ gag-specific T-cell responses are induced by DNA/Ad5 and Ad5/Ad5 vaccination. Table 1 lists a total of 120 subjects from phase I randomized trials in the United States who received the DNA/Ad5 or Ad5/Ad5 vaccination regimen; the subjects received the Ad5 vaccine at doses no lower than 1010 viral particles. The selection process was random and conducted regardless of the response status of the vaccine as defined by the IFN-γ enzyme-linked immunospot (ELISPOT) assay (11). In order to bring the size to 50, subjects for the DNA/Ad5 cohort were taken from several immunization groups which received either three or four doses of 5 mg DNA plasmid in phosphate-buffered saline or three doses of 5 mg DNA formulated with aluminum phosphate. Results of previous phase I trials indicated that the addition of the adenovirus did not improve the response rate or magnitude of the DNA vaccine, thereby supporting the combination into a single cohort (data not shown). In the end, the response rates and distribution of ELISPOT responses (as tested 4 weeks post-Ad5 boost) between the DNA/Ad5 and Ad5/Ad5 cohorts were comparable (Table 1).

Archived peripheral blood mononuclear cells collected 4 weeks after the vaccine boost from all the subjects were analyzed by multicolor flow cytometry. An example of the flow cytometric data analysis is depicted in Fig. 2; final results are summarized in Fig. 3 to 5. Figure 3 shows the percentages of the CD8+ and CD4+ vaccine responders that display a positive response to any one or more of the five markers. This figure displays an overview of the functionality of the responses and only includes subjects that responded to the vaccine. Figure 4 shows the actual distribution of T-cell subsets that were positive across all of the subjects, and Fig. 5 shows the relative frequencies of the total T-cell subsets for the DNA/Ad5 and Ad5/Ad5 cohorts. The data shown in Fig. 5 are averages of all absolute mock-subtracted values, regardless of the positivity status. Hence, the data must at least be viewed in conjunction with a nonresponder group in the same graph to differentiate response magnitudes from those of the normal assay backgrounds. We observed that 36% of the DNA/Ad5 vaccinees with positive responses to at least one of the CD8+ subsets (n = 14) exhibited antigen-specific CD8+ T cells which were a “5+” highly functional population (Fig. 3A). The majority of the responding subjects in the DNA/Ad5 and Ad5/Ad5 groups

**FIG. 3.** Percentage of vaccine responders with multifunctional T-cell responses. Graphs display the percentages of the responders in each cohort that had CD8+ (A) or CD4+ (B) T-cell populations that expressed 5, 4, 3, 2, or 1 of the markers as measured in the multi-ICS assay. All functional subsets that contained at least one positive response are defined beneath the graphs in accordance with the number of markers that were expressed in each. Individual vaccine responders often have more than one positive subset and therefore will be included in more than one category (5, 4, 3, 2, and 1).
exhibited a subset of gag-specific T cells which simultaneously expressed CD107a, IFN-γ, MIP-1β, and TNF-α+ ("4+") (Fig. 3A and 4A and B). This T-cell subset dominated the CD8+ responses in each vaccine recipient regardless of the regimen (Fig. 5A). The gag-specific CD8+ T cells which produced IFN-γ, IL-2+, MIP-1β+, and TNF-α+ (Fig. 4B) were detected in some of the DNA/Ad5 recipients. While these data demonstrate that the vaccines induce highly functional (that is, 5+ or 4+) CD8+ responses, the profiles induced by the two regimens are different.

Multifunctional CD4+ gag-specific T-cell responses are induced by DNA/Ad5 and Ad5/Ad5 vaccination. Next we performed a similar analysis to characterize the antigen-specific CD4+ T-cell functional responses to the HIV CAM-1 gag immunogen. Over 80% of the responders in both cohorts exhibited CD4+ cells which were positive for at least three of the markers being probed (Fig. 4). Double-positive gag-specific CD4+ T cells (2+) were also detected in the responding subjects, most frequently of the

FIG. 4. HIV gag-specific T-cell response profiles. A matrix grid relationship depicting the multifunctional response profiles for Ad5/Ad5 (A) and DNA/Ad5 (B) vaccine cohorts is shown. Data from the CD8+ subsets are depicted on the left matrix, and the data from the CD4+ subsets from the same subjects are aligned to the right. A gray square indicates that a positive HIV-gag response was detected. Positive responses were not detected in all 31 subsets; therefore, these matrices only display the functional subsets in which a positive response was detected from at least one of the subjects tested. The percentage of the total cohort scoring positive for each functional subset is displayed in a bar graph aligned below each matrix. The T-cell subsets marked with an asterisk contain a significantly greater number of positive responses than the adjacent cohort (graph A versus B) in the graph set (P = 0.02). Subset abbreviations are defined as follows: C, CD107a; G, IFN-γ; I, IL-2; M, MIP-1β; T, TNF-α (where a listed marker indicates that the defined subset expresses that marker, and is also negative for those that are not listed). For example, GIMT is the abbreviated notation for CD107a+ IFN-γ+ IL-2+ MIP-1β+ TNF-α+.
IFN-γ+ TNF-α+ or IL-2+ TNF-α+ cell phenotype (Fig. 4). However, within each subject, the triple-positive subset dominated the helper responses (Fig. 5B). The presence of this specific multifunctional 3+ T cell subset had been correlated with effective control of other pathogens, such as Leishmania major, IFN-γ and TNF-α possess antiviral activity and direct effector function, while IL-2 has the potential of enhancing the proliferative capacity of the T cells.

CD4 and CD8 T-cell profiles are different between Ad5/DNA and Ad5/Ad5 regimens. It is worth noting that for the DNA/Ad5 recipients, more responses were detected in a number of T-cell subsets which expressed IL-2 as a component of the response compared to that in the Ad5/Ad5 vaccinees. In fact, there were more positive responses detected in each of the CD4+ and CD8+ subsets expressing IL-2 in the DNA/Ad5 group compared to the Ad5/Ad5 group (Fig. 4). Cumulatively, there were statistically more CD8 (P < 0.00001) and CD4 (P = 0.00007) responses in subsets containing IL-2 expression in the DNA/Ad5 group. Furthermore, when directly comparing each IL-2-inclusive subset using a likelihood ratio test, both the CD4+ and CD8+ subsets of the phenotype IFN-γ+ IL-2+ MIP-1β+ TNF-α+ contained more responses in the DNA-prime cohort (P = 0.02) (Fig. 4B). To explore whether the trend of increased IL-2 expression was associated with an increase in proliferative cell responses from the DNA/Ad5 group, carboxyfluorescein diacetate succinimidyl ester proliferation assays were performed after a 7-day stimulation with the HIV gag peptide pools. The Ad5/Ad5 and DNA/Ad5 groups showed similar levels of proliferation using this assay (data not shown); therefore, we could not conclude that the DNA prime increased the proliferative capacity of the responding T cells.

The total CD4+ responses from the DNA/Ad5 group were significantly different and greater in frequency than those detected from the Ad5/Ad5 group (P = 0.02). These results also demonstrated that 50% of the DNA/Ad5 cohort displayed a positive response in one or more CD4+ subsets, compared to 34% of the Ad5/Ad5 cohort. Further evidence for the shift toward increased CD4+ responses with a DNA prime was observed by the examination of the T-cell profiles of the DNA/ Ad5 group prior to the Ad5 boost. This profile of responses as measured 4 weeks after the final DNA prime demonstrated a significant percentage of subjects with CD4+ responses; however, the CD8+ response profile was not significantly different (P = 0.02) than the HIV-seronegative group that was tested for assay false-positive rates (data not shown). Collectively, these data suggest that DNA priming increases the CD4+ component of the response and that this type of response persists after an Ad5 boost.

Multifunctional Nef and Pol-specific T-cell responses are detected in trivalent (Ad5 gag/pol/nef) recipients. The functionality and phenotypes of the Nef- and Pol-specific responses in a subset of the 70 subjects in the Ad5/Ad5 cohort who received a trivalent vaccine regimen consisting of 3 × 10^10 virus particles/dose of Ad5 gag/pol/nef (Table 1) were examined. The CD8+ Nef and Pol profiles also demonstrated the presence of highly functional responses. Over 80% of the responding subjects displayed positive CD8+ T-cell responses from subsets that expressed four markers (Fig. 3A). In concurrence with the HIV gag Ad5/Ad5 profile (Fig. 4A), the two most commonly detected CD8 phenotypes in both the Nef and Pol profiles were CD107a+ IFN-γ+ MIP-1β+ TNF-α+ followed by IFN-γ+ MIP-1β+ TNF-α+ (Fig. 6). The CD4+ T-cell responses to Nef and Pol stimulation also displayed a similar response pattern as the HIV gag-specific responses. Over 60% of the responding subjects had responses in the 3+ subsets (Fig. 3B). Specifically, the most frequently detected subset in the Nef and Pol profiles was IFN-γ+ IL-2+ TNF-α+ (Fig. 6).

**DISCUSSION**

After the recent demonstration of lack of efficacy in the STEP test-of-concept phase IIB HIV trial, many questions have arisen as to the cause of the study outcome. Indeed, there are many confounding factors which are currently being explored. There has been general agreement that the Merck candidate used in the STEP trial showed good immunogenicity as measured in a validated ELISPOT assay; however, questions have arisen regarding the quality of these T-cell responses. There has also been debate concerning the benefit of using a DNA prime to enhance the quality of the T-cell responses, but head to head comparisons of these immunological data have not been performed with the same immunogens using human clinical samples. Here we present an evaluation of the T-cell response profiles from a cohort of clinical trial subjects who received the same HIV-1 CAM-1 gag insert delivered by either a DNA prime/Ad5 boost method or an Ad5 prime/Ad5 boost immunization regimen. To our knowledge, no other group has made this direct comparison between an Ad5/Ad5 and DNA/Ad5 regimen in phase I clinical trials, and the information generated in this study should be valuable to the field of HIV vaccine research.

Recent advances in multicolor flow cytometric technology have made it possible to probe the simultaneous production of several markers on a cellular level for effector function and understand how these properties may correlate with control of pathogens. Here we describe a comprehensive qualification of the multicolor flow cytometry assay which quantifies T-cell functional markers (IFN-γ, CD107a, TNF-α, IL-2, and MIP-1β). We established assay positivity criteria (>90 events per million lymphocytes and ≥3-fold over mock) which provided an observed false-positive rate of approximately 0 to 2% per subset against the 15-mer peptide pools. These observed low false-positive rates provide confidence in the ability to use a single positivity cutoff for all subsets. This method for defining positivity is attractive because of the ease with which it can be

FIG. 6. HIV Nef and Pol-specific T-cell response profiles. A matrix grid relationship depicting the multifunctional response profiles for Nef (A) and Pol (B) from a cohort of 39 subjects vaccinated with a trivalent Ad5 gag/pol/nef is shown. Data are arranged as described for Fig. 4. Pol responses were combined for the Pol-1 and Pol-2 peptide pools; a subset was marked as positive if the positivity criterion was met for one or both peptide pools. The percentage of the total cohort scoring positive for each functional subset is displayed in a bar graph aligned below each matrix.
used by operators to assign result status on the basis of the assay results. Furthermore, this represents, to our knowledge, the first assay qualification that was published for a sizeable flow cytometric assay. This study further extends the eight-color ICS assay validation work of H. Horton, et al. (14), who primarily focused on two of the T-cell measurements, IL-2 and IFN-γ. In the present analysis, a full set of data output from five markers in the CD8+ and CD4+ subsets was measured, yielding 62 results per stimulation condition. This complex data output captures the full power of flow cytometric analysis because each cell is categorized into a specific phenotypical subset, yielding very detailed information about the quality of the immune response.

Antigen-specific CD8+ cells capable of simultaneously displaying multiple T-cell functionalities (degranulation, IFN-γ, MIP-1β, TNF-α, and IL-2 production) have been associated with HIV nonprogressors (4) and have been detected in protective immune responses from vaccinia virus vaccination (24) as well as from AIDS-related cytomegalovirus retinitis (IFN-γ and IL-2) (27). Moreover, the contribution of multifunctional CD4+ T-cell responses has recently been shown in HIV nonprogressors (16) and has been correlated with protection from *Leishmania major* and *Mycobacterium tuberculosis* in a mouse model (10). Collectively, these data indicate that multifunctional T-cell responses are desirable.

Here, we report that both DNA/Ad5 and Ad5/Ad5 vaccines are capable of inducing multifunctional CD4+ and CD8+ T-cell responses to HIV-1 gag, yet the profiles appear to be different between the regimens. A majority of the DNA/Ad5 and Ad5/Ad5 vaccine responders exhibited CD8+ responses of the CD107a+ IFN-γ+ MIP-1β+ TNF-α+ phenotype, and this subset represents the highest frequencies found of the total CD8+ T-cell response of each cohort. However, this non-IL-2-expressing subset has never been uniquely associated with reduced disease burden. The presence of a few gag-specific responses in the CD8+ 5+ subset of cells was detected in both cohorts. This population was detected at a higher frequency of subjects from the DNA/Ad5 group, yet this difference did not reach statistical significance. Other IL-2-expressing CD8+ subsets, such as IFN-γ+ IL-2+ MIP-1β+ TNF-α+ and IFN-γ+ IL-2+ TNF-α+ populations were observed only in subjects who received the DNA/Ad5 vaccination. CD4+ multifunctional cells were also present; most frequently found in both groups was the 3+ phenotype of IFN-γ+ IL-2+ TNF-α+. This particular triple-producing cell type has been shown to be functionally superior to single-cytokine-producing cells; coincident expression of IL-2 and IFN-γ in HIV-1-specific helper cells has been associated with control of viral replication in infected individuals (5, 12). Furthermore, these cells have been shown to produce more cytokine per cell (15). Clearly, the DNA/Ad5 regimen induces a more diverse set of phenotypes, particularly with the enhanced presence of IL-2-expressing subsets. For example, there were significantly more responses in subsets containing IL-2 expression from both the CD8+ and CD4+ cells in the DNA/Ad5 group than the Ad5/Ad5 group. The analysis of the Nef and Pol specific responses in recipients of the trivalent vaccine consisting of Ad5 gag/pol/nef yielded profiles which were consistent with that of gag delivered by the same regimen.

The data set presented here illustrate a few more notable differences in the T-cell response profiles induced by DNA/Ad5 and Ad5/Ad5 vaccine regimens. The Ad5/Ad5 profile exhibits a more even CD8+/CD4+ distribution than that of the DNA/Ad5 cohort. Overall, 27 (39%) and 24 (34%) of the 70 subjects had one or more positive subsets in the CD8+ and CD4+ population, respectively; 14 of 70 (20%) had both detectable CD4+ and CD8+ subsets. In contrast, 14 (26%) and 25 (50%) of 50 DNA/Ad5 subjects had positive subsets in the CD8+ and CD4+ populations, respectively; 9 of the 50 (18%) had detectable responses in both CD4+ and CD8+ populations. We observed that 16 (32%) of 50 DNA/Ad5 subjects had detectable CD8+ subsets with no gag-specific CD8+ T cells, compared to only 10 (14%) of the Ad5/Ad5 subjects. Clearly, the DNA/Ad5 method resulted in significantly more CD4+ than CD8+ gag-specific responses when comparing the two T-cell subsets within the cohort (P = 0.01). The DNA/Ad5 group also had significantly more CD4+ responses overall compared to the Ad5/Ad5 group (P = 0.02). At the present time, it is not known how HIV-specific CD4+ responses may alter the outcome of an HIV-1 prophylactic vaccine trial. Such responses could provide immunological help and increase proliferative responses or, conversely, could provide an unwanted detrimental effect by serving as a prime target for HIV viral infection.

In summary, a comprehensive evaluation of the T-cell response profiles from clinical trial subjects who received the same HIV CAM-1 gag insert delivered by either a DNA prime/Ad boost method or a Ad5/Ad5 method shows that both of these HIV vaccine candidates elicit multifunctional CD4+ and CD8+ T-cell responses. Certain distinctions exist between the two profiles, including trends toward increases in antigen-specific CD4+ responses, IL-2 expression, and diversity in phenotypes in the DNA/Ad5 cohort. It is unclear from this study how preexisting vector immunity influenced the T-cell profiles; the phase I trials did not involve prestratification on the basis of their preenrollment Ad5 immunity and hence are unable to address this factor. To date, there has been no definitive evidence from nonhuman primate challenge studies that these discriminating properties contribute to vaccine efficacy. We showed previously that the DNA/Ad5 regimen delivering a simian immunodeficiency virus (SIV) gag antigen was more efficacious than the Ad5/Ad5 regimen against a SIVmac239 challenge (7), but the outcome was readily correlated with the magnitude of the vaccine-induced gag-specific responses. This begs the question if the potential utility of the DNA/Ad5 immunization over Ad5/Ad5 may instead be in inducing higher levels of immunity. However, our phase I studies indicated that both regimens induced comparable levels of responses to the same antigen (data not shown) and that even in individuals with high preenrollment immunity to Ad5, the differences in vaccine response rate using both regimens were minimal. The disparity in vaccine-induced immunity in nonhuman primate and human testing was not unexpected, however. In conclusion, the main distinctions between both regimens are the following: (i) the phenotypes of the induced T-cell responses which may influence the potency of the vaccine and (ii) the extent of exposure to the Ad5 vector which may factor into the safety of the immunization regimen. At this time, these statements are theoretical considerations that a DNA prime could fare differently in a proof-of-efficacy clinical trial and it remains
a challenging decision for the research community as to whether a heterologous DNA prime-adenovirus boost regimen warrants additional clinical testing.

ACKNOWLEDGMENTS

We thank Michael Betts for helpful discussions concerning multicolor flow cytometric analysis and LSRII instrumentation and Mario Roederer for providing the SPICE analysis software. We also acknowledge Timothy Tobey for advice given during early assay development. We extend our appreciation to the volunteers and staff who conducted these studies at their respective sites.

This work was supported by funding from Merck & Co., Inc.

REFERENCES


