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Short communication

Induction of HIV-specific functional immune responses by a multiclade HIV-1 DNA vaccine candidate in healthy Ugandans

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Abstract

A phase I randomized, double blind, placebo-controlled trial to assess the immunogenicity of a multiclade HIV-1 DNA plasmid vaccine was conducted in 31 HIV-1-negative Ugandans. Following immunization with DNA at 0, 1, and 2 months, the frequency of HIV-specific immune responses was assessed up to 10 months using a standard chromium release assay (CRA), lymphoproliferative assay (LPA), and antibody dependent cell-mediated cytotoxicity assay (ADCC). Seven of 15 (47%) vaccinees demonstrated CTL activity using the CRA to HIV-1 Env B with responses observed 1 month following the second vaccination and as late as 7 months following complete immunization. Additionally, lymphoproliferative reponses were observed in 14/15 vaccinees against p24. No CTL or LPA responses were observed at baseline or in the placebo group. ADCC activity was minimally induced by DNA vaccination. This study demonstrates that immunization with DNA alone induces CTL and lymphoproliferative responses in a population that will participate in a phase IIb study evaluating HIV-1 DNA priming followed by boosting with a replication-defective recombinant adenovirus vector.

Keywords: DNA vaccine; HIV-1; CTL; LPA; ADCC

1. Introduction

In 2005, an estimated 38 million people worldwide and 1 million Ugandans were living with HIV-1 [1]. A disproportionate burden of HIV-1 resides in sub-Saharan Africa, where the population is insufficiently prepared to combat the effects of this epidemic. Although access to antiretroviral drugs is increasing in resource limited settings and the incidence of HIV-1 infection is declining in most parts of the world, a preventive vaccine is essential for more effective pandemic control. Viral genetic heterogeneity poses a major hurdle in developing an HIV-1 vaccine, especially in Africa, where a majority of the HIV-1 genetic diversity is found [2]. In East Africa alone, three different subtypes (A, C, and D) predominate in addition to a growing number of circulating recombinant forms [2,3]. Multiclade vaccine constructs consisting of HIV-1 subtypes A, B, and C components have been proposed as a means to cover most global HIV-1 diversity [4].

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Over one-third of HIV-1 vaccine candidates in human trials include plasmid DNA expression vector components [5]. Immune responses to HIV-1 DNA vaccines exhibit varying results. A DNA vaccine encoding the HIV-1 *env* and *rev* genes in seronegative individuals induced HIV-specific lymphoproliferative responses, and after in vitro stimulation interferon-gamma (IFN-γ), beta-chemokines and CTL activity was detected, as measured by the ^{51}Cr release assay (CRA), but the response was CD4 Th1 polarized and not durable [6]. More recently, an HIV-1 DNA vaccine proved safe but failed to elicit either CD4 or CD8 cellular immune responses [7]. DNA vaccines may be useful in HIV vaccine development through priming of humoral and cellular immune responses when used in combination with recombinant vectors or proteins [8]. In measuring immune responses to a modified vaccinia Ankara (MVA) vectored HIV vaccine with or without DNA priming, T-cell responses were enhanced in those recipients of a DNA vaccine prime [9].

The vaccine strategy developed by the Vaccine Research Center (VRC), NIAID, NIH, Bethesda, MD, combines synthetically modified HIV-1 *env*, *gag*, *pol* and *nef* DNA representing HIV-1 subtypes A, B and C, the predominant subtypes found in Africa, the Americas, Europe and Asia. In a recently completed phase I clinical trial in the U.S., the vaccine was shown to be well-tolerated and induced cellular and humoral responses [10]. Immunomonitoring was performed using IFN-γ ELISpot and intracellular cytokine staining (ICS) assays measuring IFN-γ and/or interleukin-2 (IL-2) and showed induction of cytokines to HIV-1 Env A, Env B, and Env C peptide pools in both CD4 and CD8 T-cells [10]. The ELISpot and ICS assays are sensitive assays for detecting cytokines, chemokines, and markers of degranulation, but do not provide direct information on the lytic activity of T-cells on targets expressing HIV-1 antigens, which is the goal for a vaccine-induced CD8$^+$ T-cell. The objective of this study was to determine the frequency and duration of HIV-specific functional immune responses in Ugandan HIV-1 seronegative recipients of a multiclade HIV-1 DNA Vaccine (VRC-HIVDNA009-00-VP).

2. Methods

2.1. Vaccine

VRC-HIVDNA-009-00-VP (Vaccine Research Center, NIH, Bethesda, Maryland) is a vaccine composed of four DNA plasmids encoding proteins from HIV-1 [10]. The DNA plasmid VRC-4306 is designed to express HIV-1 polyproteins (structural core protein Gag, viral polymerase Pol, and accessory protein Nef) from clade B and is 50% (by weight) of the vaccine. The DNA plasmids VRC-5305, VRC-2805, and VRC-5309 express the HIV-1 Env glycoproteins from clades A, B, and C, respectively, and are each 16.67% (by weight) of the vaccine.

2.2. Study design

The primary objective of this protocol was to study the safety and tolerability of VRC-HIVDNA-009-00-VP at a dose of 4 mg administered intramuscularly using a needle-free injection system (Biojector®, Bioject Incorporated, Bedminster, New Jersey) at 0, 4, and 8 weeks in the same deltoid muscle. Thirty-one Ugandan low risk for HIV, healthy subjects were initially enrolled in this phase I randomized, double blind, placebo-controlled trial and followed for 11 months. Volunteers were randomly assigned into two groups. Group A ($n = 16$) received phosphate buffered saline placebo and group B ($n = 15$) received HIVDNA009-00-VP. Monitoring for HIV-1 infection was performed using a sequential algorithm of the following U.S. FDA approved tests: Systems rLAV ELISA (BioRad Laboratories, Redmond, WA), Vironostika HIV-1 Microelisa (bioMerieux, Durham, NC) and rLAV HIV-1 Western Blot Kit (BioRad Laboratories) and Amplicor HIV-1 Monitor version 1.5 RT- PCR (Roche Diagnostics, Indianapolis, Indiana).

2.3. Cytotoxic T lymphocyte assay

A standard CRA on freshly isolated peripheral blood mononuclear cells (PBMC) was performed, as previously described [11]. Briefly, PBMC were isolated from acid citrate dextrose anticoagulated whole blood within 6 h of collection using Leucosep® tubes (Greiner Bio-One, Germany). In vitro stimulations were set up using 20 million freshly isolated PBMC, of which 20% of the PBMC (stimulators) were infected with a vaccinia recombinant expressing HIV subtype B (IIIB) *env*/*gag*/*pol* genes (vABT489) at a multiplicity of infection of five plaque-forming units per cell for 1.5 h. Cultures were initiated with IL-7 (330 U/ml; Sigma–Aldrich, Germany) and boosted after 7 days with IL-2 (20 U/ml; Sigma-Aldrich, Germany). Cultures were maintained for a total of 14 days. Autologous EBV-transformed B lymphoblast cell lines (BLCL) were generated for each enrolled volunteer. Prior to the CTL assay, BLCL were infected overnight with vaccinia recombinants expressing subtype B (BH8) *env* (vPE16), subtype B (pHXB2) *gag* (vDK1,), or empty vector (vSC8), and labeled with ^{51}Cr for use as targets in the CTL assay. Cold target empty vector infected BLCL were used to compete and absorb background vaccinia responses. All vaccinia constructs were obtained from the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH and expanded by Advanced BioScience Laboratories, Inc. (Rockville, MD).

CTL assays were performed at two effector:target ratios of 50:1 and 25:1, with whole, CD8 or CD4-depleted PBMC. CD8 and CD4 depletions were performed using Dynal (Invitrogen; Carlsbad, California) magnetic beads, as described previously [11], in order to verify CD8 restriction.

2.4. Lymphoproliferative assay

The proliferative responses of subject PBMC were measured by incubating 1×10^5 cells per well in 96-well U-bottom plates with serial antigen concentrations (10, 5, 1 μg/ml) of subtype B $p24_{LAI}$ (ABL Inc., Kensington, MD) and 10 μg/ml of subtype B envelope protein $gp160_{451}$ (ABL Inc., Kensington, MD); tetanus toxoid (Staten Serum Institute, Copenhagen, Denmark) was used at 5 μg/ml as a recall antigen. In a separate plate, PBMC were also cultured with 2 μg/ml of PHA (Sigma St. Louis, MO). After 3 days of incubation with PHA and 6 days with the antigens, cells were pulsed with 1 μCi/well of [^{3}H]-thymidine for 6 h, then harvested using the Filtermate 196 Harvester (Packard Bioscience Company, Meriden, CT) and counted in a TopCount NXT™ microplate scintillation and luminescence counter (Packard Bioscience Company, Meriden, CT).

2.5. Antibody dependent cell-mediated cytotoxicity assay

The ADCC assay was performed as previously described [12]. Briefly, sera were collected at visit 3 (pre-vaccination) and visit 11 (2 weeks post third vaccination) from 14 placebo recipients and 15 vaccine recipients. PBMC from Thai HIV-seronegative donors, were used as effector cells. CEM.Nk^r cells obtained from the NIH AIDS research and reference reagent program, Division of AIDS, NIAID, NIH, were used as targets.

CEM.NK^r cells were cultured overnight and labeled with ^{51}Cr (Perkin Elmer Life and Analytical Sciences, Boston, MA, USA) at 100 μCi per 1.0×10^6 cells/ml and incubated with 1 μg of HIV-1 envelope protein, gp120 IIIB (Advanced Biotechnologies Inc., Columbia, MD, USA). Sera for measurement of ADCC were thawed and heat inactivated for 30 min at 56° ± 2 °C, and used at the following dilutions: 1:100; 1:1000; 1:10,000 and 1:100,000.

Target cells were incubated with serum for approximately 20 min at room temperature (25 ± 5 °C). Thawed and overnight-rested PBMC were added to all wells containing either serum or plasma and targets at an effector:target ratio of 100:1, and the mixture was incubated for 6 h. Sodium dodecyl sulphate (SDS) (Gibco BRL, Grand Island, NY, USA) was added to target cells to assess maximal chromium release, while untreated target cells were used to establish spontaneous chromium release. Chromium release was measured using TopCount NXT™ (Packard Bioscience Company, Meriden, CT, USA) and percentage of specific lysis (%SL) of target cells from each serum dilution was calculated.

2.6. Data analysis

All assays were conducted on blinded samples. For the CRA, specific lysis was calculated as [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100. Background vaccinia responses were subtracted from HIV-1 specific responses. A positive CTL was defined as specific lysis greater than 10% at either effector to target ratio, provided that elevated lysis at 25:1 was supported by lysis at 50:1. The response must have shown at least 50% reduction in lysis in the CD8 depleted effector group, in order to be considered CD8 specific (Fig. 1). For the lymphoproliferative assay, the data are expressed as a lymphocyte stimulation index (LSI) = [(PBMC cpm + antigen/mitogen)/(PBMC cpm + medium)] to define antigen specificity. Individuals are arbitrarily designated as responders or non-responders if their LSI is greater than or equal to five. For the ADCC, sera from each volunteer were assessed in parallel with the plasma controls in the same assay plate. Maximum percentage of specific lysis at visit 11 (2 weeks post-completion of vaccination) was subtracted from that at visit 3 (pre-vaccination), the baseline ADCC activity to gp120 for each subject. Maximum percentage of specific lysis for the HIV-positive plasma control was calculated by subtracting the amount of lysis in HIV-negative control plasma to reduce inter-assay variability. Results of the ADCC assay from vaccinees are expressed as percent relative lysis (%RL) to the positive-control %SL and were calculated. Results were analyzed by using the maximum % RL for each volunteer.

Descriptive statistics (mean, median, standard deviation) were used to evaluate the distribution of responses among placebo and vaccinees. In addition, we compared groups using a dichotomous outcome (positive or negative response) using the 90th percentile of the % relative response among controls for the antigen as the cutoff for a positive response. Although, this is somewhat arbitrary, this cutoff was chosen *in lieu* of using a multiple of the standard deviation, since none of the distributions approximated the normal. Fisher's

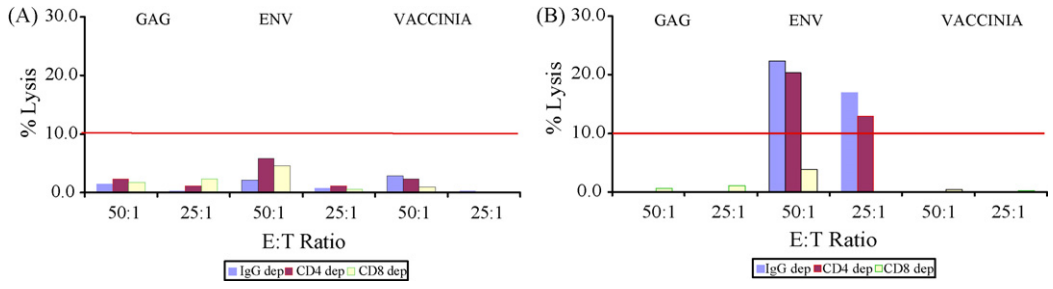

Fig. 1. Example of negative (A) and positive (B) CTL responses.

Table 1
Frequency of CD8 cytotoxic T lymphocyte responses to human immunodeficiency virus type 1 Env subtype B (IIIB) among recipients of HIVDNA 009-00-VP

Time	Point frequency: no. positive/no. tested (%)	Cumulative frequency: no. positive/no. vaccinated (%)
0 (pre-vaccine)	0/8	0/15
1 month post 2nd vaccine	2/10 (20)	2/15 (13)
1 month post 3rd vaccine	1/12 (8)	3/15 (20)
4 months post 3rd vaccine	0/12 (0)	3/15 (20)
7 months post 3rd vaccine	5/12 (42)	7/15 (47)
10 months post 3rd vaccine	0/13 (0)	7/15(47)

Note: 0/14 placebo recipient demonstrated a positive response.

exact test was then used to test statistical significance between groups.

3. Results

Vaccinations were completed in 29 participants, all of whom completed 48 weeks of follow-up. Overall, vaccinations were tolerated, with mild to moderate reactogenicity reported. There were no episodes of severe reactogenicity. There were no HIV-1 seroconversions during the study.

In vitro stimulations were completed on 135 of 186 (73%) samples and the CRA performed. No HIV-specific CTL responses were observed at baseline or in the placebo arm for the duration of the study. No *gag* specific HIV-1 responses were observed. All positive CTL responses were CD8-restricted and directed solely against envelope antigen. HIV-1 specific responses were observed in 2 of 10 (20%) volunteers as early as 1-month after the second vaccination. The greatest number of positive CTL responses at one time point, 5 of 12 (42%), occurred 7 months following the third injection (Table 1). Five volunteers demonstrated a response as late as 7 months, following the third immunization. HIV-1 envelope-specific cumulative CTL responses were seen in 7 of 15 (47%) vaccine recipients (Table 1), of which 1 of 15 (6.7%) volunteers had positive responses at multiple time points. This volunteer had responses at 1 month post the 2nd vaccination and again at 7 months post the 3rd vaccination. The magnitude of specific lysis in the CRA ranged from 11.2–24.7%.

LPA evaluations were done on samples drawn at baseline and 1 month after the third injection. Analysis was done using both the as-treated and intent-to-treat methods. The as-treated method includes only fully immunized participants. The intent-to-treat method includes data from all enrolled volunteers who were not replaced regardless of how many study injections they received. For each vaccine/placebo group at each visit, minimum, maximum and median LSI values were calculated. Chi-square and Wilcoxon rank tests were performed for response by vaccine/placebo group and visit. There were no differences in the as-treated and intent-to-treat group analysis. Responses to gp160 in the vaccine group at baseline were 0/15 and 1/15 at visit 12. Responses to p24 in the vaccine group at baseline were 0/15 and 14/15 at visit 12. Placebo responses to gp160 and p24 were 0/14 at visit 12 (Table 2). Responses to the recall antigen, tetanus toxoid, were 21 and 33% in the placebo group at baseline and V12, respectively, and were 33 and 47% in the vaccine group at baseline and at visit 12, respectively. All volunteers tested responded to mitogen at baseline and V12. Ugandans are routinely vaccinated as children against tetanus, however, boosting is rarely performed unless injury occurs. Unfortunately, we do not have information on the volunteers in this study regarding tetanus immunization. The lower response rate (21–47%) is likely due to the lack of booster vaccinations after childhood.

The ADCC assay was performed in parallel between each vaccinee's serum and the HIV-positive plasma control in the same assay plate. The average %SL for the positive plasma control samples ($n = 29$ assays) was $40.5 \pm 5.9\%$, and the median was 39.7%. Among placebo recipients ($n = 14$), the average ADCC response expressed as %RL was $1.4 \pm 2.2\%$, and the median was 0.0% with an interquartile range of 2.0%. The average HIV-specific ADCC activity expressed as %RL among vaccine recipients ($n = 15$) was $3.2 \pm 4.0\%$, the median was 1.9% with a interquartile range of 4.6% (Data not shown). Using the 90th percentile response in the placebo group, there were 1/14 placebo responders and 3/15 vaccinee responders, giving a p value of 0.60 by Fisher's exact test.

4. Discussion

The vaccine candidate VRC-HIVDNA009-00-VP was found to be well-tolerated and not related to any serious

Table 2
HIV-specific lymphoproliferative responses 4 months after vaccination

Antigen	Placebo					Vaccinees				
	N	# Positive (%)	Median LSI	Minimum LSI	Maximum LSI	*N*	# Positive (%)	Median LSI	Minimum LSI	Maximum LSI
$gp160_{451}$	15	1 (6.67)	1	0	21	15	1 (6.7)	1	0	5
$p24_{LAI}$	14	0 (0)	1	1	3	15	14 (93.3)	11	1	24

adverse events in Ugandans. This same product showed promising T-cell immunogenicity in U.S. volunteers as evidenced by CD4 and CD8 ICS studies [10]. Positive CD8 T-cell responses directed against vaccine antigens were detected in 40% of vaccinees by ICS, as measured by IFN-γ and/or IL-2 production and were sustained [10]. The Ugandan trial extends the findings of the U.S. study with this DNA vaccine to other functional immunogenicity assays. VRC-HIVDNA009-00-VP induces functional CD8 CTL activity measured by CRA against HIV-1 Env-B vaccine antigens as early as 1 month following the second vaccination. HIV-specific CD8 T-cell responses in the U.S. study were also detected after the 2nd immunization using ICS, and the response was also exclusively to HIV-1 Env-specific peptides pools. A recently published trial in which volunteers were immunized with up to 3 mg of DNA vaccine construct containing 6 HIV genes failed to elicit T-cell responses as detected by either and IFN-g ELISPOT assay or CRA. [7] The VRC-HIVDNA009-00-VP construct did induce CTL responses and therefore, appears to be more potent. DNA is not typically used as a stand alone vaccine and boosting with viral vector vaccines will, most likely, significantly improve immune responses. In addition, DNA plasmids induce more CD4 than CD8 T-cell responses, while adenoviruses preferentially stimulate CD8 T-cell responses. [13] In ongoing studies, boosting with adenovirus constructs significantly improved T-cell responses, in particular CD8 T-cell responses [10]. The time-point with the greatest CTL activity in the Uganda study was 7 months following the third injection, whereas the frequency and magnitude of the $CD8^+$ response measured by ICS was 4 weeks after the 3rd vaccination. The detection of CTL responses at 7 months post-immunization in this trial is similar to findings with Canarypox vectors [11]. Persistent CTL responses are desirable and highlight the need for continued testing at multiple time-points with the CTL and other assays. In the current study, 7 of 15 (47%) vaccinees had detectable HIV-specific CD8 restricted T-cell responses measured by the CRA which is comparable to the 40% frequency of positive CD8 T-cell responses observed by ICS in the U.S. trial. One major difference in the immunogenicity data, from the U.S. versus the Ugandan trial, was the ability in the former to detect sustained responses at consecutive time points throughout the study. For the CRA, only 1 of 7 positive responders was positive at multiple time points. The detection of sustained vaccine-induced immune responses in the U.S. study may be due to the increased sensitivity of the ICS assays, to the peptides used or other methodological differences compared to the CRA. Others have reported greater sensitivity of an in vitro expanded ELISPOT compared to the CRA in HIV-1 vaccine trials [14]. We were not able to examine responses to all three Env antigens because of the lack of availability of vaccinia constructs matching the Env A and C antigens in the vaccine constructs and also due to the need to set up more than one IVS which would not have been feasible due to the additional blood volume required. Graham et al. reported strong responses to the Env A, which we were not able to examine in our studies [10].

The lymphoproliferative responses to HIV p24, but not HIV-1 gp160, were robust, implying vigorous HIV-specific $CD4^+$ T-cell responses. However, the antigen profile of the responses differed from the U.S. trial. The U.S. trial data showed a greater frequency of CD4 T-cell responses to HIV Env (95%) versus Gag (15%). However, it should be stressed that the Uganda trial measured PBMC proliferation, rather than CD4 T-cell cytokine secretion. In addition, the stimuli between assays were markedly different (protein versus peptide pools) and the U.S. study used peptides identical to that used in the vaccine. We do not believe that direct comparisons can be made between the two assay platforms. A similar difference has been noted when using proteins versus peptide pools [15].

As the development of a safe and effective HIV-1 vaccine continues, defining correlates of protection becomes more important. It has been speculated that an effective HIV-1 vaccine will ultimately need to generate broadly neutralizing antibodies and a robust cellular immune response [16]. Although HIV-specific $CD8^+$ T-cells may not prevent infection, they are thought to be important due to their positive association with long-term survivors, highly exposed persistently seronegative individuals and in the virologic control of primary infection [8]. Recently, a considerable amount of progress has been made in the standardization of cellular immunogenicity assays to address vaccine-induced immune responses, particularly for ELISpot and ICS. Although there are newer assays that use surrogate markers of cytolysis, the CRA continues to be the gold standard for measuring functional cytolysis [17]. The CRA remains a standard part of immunogenicity testing for the U.S. Military HIV Research Program, as it was the assay used from the inception of vector-based HIV-1 vaccine trials, thus allowing comparisons of immune responses from different vaccine strategies [18].

The clinical trial pipeline for HIV-1 vaccines continues to expand as new targets for immunity are discovered and more immunogenic candidates are manufactured. As more trials are conducted, it is imperative to compare how vaccines induce immune responses using consistent techniques when possible. One vector extensively used in human trials is the canarypox vaccine, ALVAC, expressing different HIV-1 genes. In ALVAC vaccine trials using HIV-1 seronegative individuals, conducted throughout the U.S., 30–60% cumulative CTL responses were observed in different studies [18]. In Thailand, ALVAC vaccine (vCP1521) expressing multiple HIV-1 genes (CRF01_AE *env* and subtype B *gag* and *pol*) induced a cumulative CTL response of 24% as measured by CRA to both Env and Gag/Pol antigens [11]. The cumulative responses were 16% to the Env and 11% to the Gag/Pol and showed persistence in 41% of the responders [11]. In Uganda, the canarypox vector expressing subtype B genes (*env*, *gag*, and *pro*) was given to 20 HIV-1 uninfected participants and 20% developed CRA CTL responses to Gag and Env with 10% being specific to Env and 10% specific to Gag

[14]. Although the size of this initial trial was small, cumulative CTL responses induced by DNA were higher than those seen with ALVAC trials in Thailand and Uganda. Additionally, ADCC responses generated by DNA, while minimal, were comparable to ALVAC administered alone as reported in Thailand [12].

VRC-HIVDNA009-00-VP is capable of inducing HIV-specific CTL activity and lympohoproliferation, and is promising as a potential vaccine candidate. These data provide additional and complementary evidence showing that this vaccine candidate can induce T-cell (presumably predominantly $CD4^+$ proliferation) and $CD8^+$ T-cells capable of lytic activity. Use of the CRA has been largely discontinued, but data from this DNA vaccine study indicate that CTL responses are of a similar or greater magnitude than those induced by canary pox vectors. Future strategies of second-generation DNA products coupled with replication-defective adenovirus vector boosting may further increase the immunogenicity of this product.

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