Safety and Immunogenicity of DNA Prime and Modified Vaccinia Ankara Virus-HIV Subtype C Vaccine Boost in Healthy Adults

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A randomized, double-blind, placebo-controlled phase I trial was conducted in 32 HIV-uninfected healthy volunteers to assess the safety and immunogenicity of 3 doses of DNA vaccine (Advax) plus 1 dose of recombinant modified vaccinia virus Ankara (MVA) (TBC-M4) or 3 doses of TBC-M4 alone (groups A and B, respectively). Both vaccine regimens were found to be safe and well tolerated. Gamma interferon (IFN-γ) enzyme-linked immunosorbent spot (ELISPOT) assay responses were detected in 1/10 (10%) individuals in group A after three Advax primes and in 9/9 individuals (100%) after the MVA boost. In group B, IFN-γ ELISPOT responses were detected in 6/12 (50%) and 7/11 (64%) individuals after the second and third MVA vaccinations, respectively. Responses to all vaccine components, but predominantly to Env, were seen. The breadth and magnitude of the T-cell response and viral inhibition were greater in group A than in group B, indicating that the quality of the T-cell response was enhanced by the DNA prime. Intracellular cytokine staining indicated that the T-cell responses were polyfunctional but were skewed toward Env with a CD4+ phenotype. At 2 weeks after the last vaccination, HIV-specific antibody responses were detected in all (100%) group B and 1/11 (9.1%) group A vaccinees. Vaccinia virus-specific responses were detected in all (100%) group B and 2/11 (18.2%) group A vaccinees. In conclusion, HIV-specific T-cell responses were seen in the majority of volunteers in groups A and B but with a trend toward greater quality of the T-cell response in group A. Antibody responses were better in group B than in group A.

An estimated 34 million people are living with HIV, and despite progress in prevention and treatment, the best long-term tool for breaking the transmission cycle is a prophylactic HIV vaccine (1–6). A vaccine with the ability to induce HIV-specific humoral and cellular immune responses that can help prevent infection and/or ameliorate disease progression should be a top public health priority. Significant advances have been made in the past several years with respect to design and development of HIV vaccines. A phase Iib trial in Thailand of ALVAC-HIV and AIDSVAX gp120 B/E prime-boost (RV144) showed that the vaccine regimen was 31.2% efficacious in preventing HIV infection during the 3.5 years of follow-up in a low-incidence largely heterosexual population (7). The RV144 volunteers were immunized four times (0, 4, 12, and 24 weeks) with a modified canarypox virus vaccine expressing HIV Gag, Pol, and Env with a concurrent boost of gp120 clade B and E proteins in adjuvant at the 12- and 24-week time points. HIV-specific CD4 T cells and antibody responses were seen in the majority of individuals, and CD8 cytotoxic T cells were induced in around a third of vaccinees (7). As a result of this pivotal study, poxvirus vectors are receiving renewed attention for delivery of tuberculosis (TB), malaria, and HIV antigens in various prime-boost combinations (5, 8, 9). Modified vaccinia virus Ankara (MVA) has been used as a candidate smallpox vaccine and has had a favorable safety profile in >100,000 humans (8, 10, 11). Multiple immunizations with MVA are tolerated, and both T-cell and antibody responses are detected in the majority of volunteers (12–16). Anti-MVA responses do not appear to significantly impair subsequent immune responses, though immune responses tend to plateau after two immunizations (12, 14, 16, 17). To circumvent antivector immunity and prime immune responses, the use of DNA as prime in “prime-boost” regimens with MVA, adenovirus, and other vector-based vaccines as the boost has become a common strategy (18–26). In these DNA prime, vector boost studies, typically polyfunctional T-cell responses, tier 1 neutralizing and nonneutralizing antibody responses, and even detection of effector T cells in the gut have been demonstrated, though responses are critically dependent on the insert, regimen, and time of sampling. In parallel, in nonhuman primates, DNA priming strategies followed by MVA have been tested extensively, and similar sustained polyfunctional T-cell responses and tier 1 neutralizing and nonneutralizing antibody responses have been demonstrated, along with partial control of pathogenic simian immunodeficiency virus (SIV)/simian-human immunodeficiency virus (SHIV) challenges (27–33). In spite of numerous SIV and SHIV challenge studies in nonhuman primates as well as the results of the RV144 study, the correlates of protection against HIV and SIV remain poorly understood (6, 34, 35). In this study, we compared the safety and immunogenicity of two clade C HIV vaccines (Advax and TBC-M4) that have previously been tested individually in...
humans. The Advax vaccine consists of two DNA plasmids administered in a 1:1 ratio via Biojector, one containing HIV-1 env and gag and one containing pol and nef (36, 37). The TBC-M4 is an MVA recombinant vaccine carrying subtype C env, gag, the reverse transcriptase (RT) gene, rev, tat, and nef (16). The purpose of the study was to characterize immune responses to the two vaccines and determine if DNA can act as a “hidden prime” and improve functional T-cell responses to MVA.

MATERIALS AND METHODS

Vaccines. Advax is a DNA vaccine designed by the Aaron Diamond AIDS Research Center (ADARC) based on the commercially available plasmid backbone pVAX1, carrying Chinese HIV-1 subtype C/B env and gag genes in one plasmid and pol and a nef/tat construct designed to express a fusion protein in the second plasmid, with plasmids mixed in a 1:1 ratio (37, 39). Advax was manufactured by Vical Inc. (San Diego, CA). Six Advax peptide pools containing the sequences of gag, rev, tat, and nef (16). The MVA candidate vaccine was formulated in a sterile phosphate saline buffer containing 0.01 M sodium phosphate and 150 mM sodium chloride. Four milligrams of Advax was administered in a total injected volume of 1 ml intramuscularly (i.m.) by Biojector (Bioject Medical Technologies Inc., Tigard, OR). The second vaccine candidate, TBC-M4, manufactured by Therion Biologics Corporation (Cambridge MA), is a recombinant modified vaccinia virus Ankara (MVA) carrying Indian HIV-1 subtype C env, gag, the reverse transcriptase (RT) gene, rev, tat, and nef (16). The MVA candidate vaccine was formulated in phosphate-buffered saline (PBS) with 10% glycerol. A dose of 5 × 10⁷ PFU was administered in a total injected volume of 0.5 ml i.m. by needle and syringe. PBS with 10% glycerol was used as a placebo for the clinical study. The amino acid sequence homology between the two constructs was more than 85% for most of the proteins (Gag, 95%; Env, 87.1%; Pol/RT, 96.4%), although it was lower for Tat (66.3%) and Nef (18.9%).

Study design. The study was a phase I randomized, double-blind (with respect to volunteer and study personnel), placebo-controlled study. Thirty-two volunteers participated and were randomized into two groups, A and B, of equal size. The study design is summarized in Table 1 and clinical trials registry NCT00902824.

Vaccines. Advax recombinant MVA (rMVA), TBC-M4 at 5 × 10⁷ PFU/dose given intramuscularly; DNA, Advax HIV-1 DNA at 4 mg/dose given intramuscularly by needle-free injection using Biojector 2000.

Safety. Local and systemic solicited adverse events (AE) (reactions) were evaluated by the study staff prevaccination, at 30 min postvaccination, and at a clinic visit 2 weeks after each vaccination, with a phone contact 7 days after the first vaccination. Volunteers also reported self-assessments between days 1 and 14 after vaccination and at other unscheduled time points if they experienced symptoms. Local reactivity events assessed were pain and tenderness, erythema/skin discoloration, skin damage (vesiculation, ulceration), induration, edema, and formation of a crust or scab. Systemic reactivity events assessed were chills, malaise, myalgia, headache, subjective fever, nausea, vomiting, arthralgia, and tiredness; temperature was recorded at clinic visits or if elevations were reported by volunteers. A physical examination was performed at every visit, and protocol-specified investigations (hematology, biochemistry and immunology, and urinalysis) were performed at screening and at the final study visit. ECG and plasma cardiac troponin I were assessed at screening, week 28, and the final study visit. Urine pregnancy testing was performed at screening, prior to each study vaccination, and at the final study visit. Unsolicited AE recorded during the trial were graded using an adaptation of the Division of AIDS (DAIDS) toxicity grading table; the relationship to the study product was assessed as not related or as unlikely, possibly, probably, or definitely related to the investigational product.

HIV testing. At screening prospective volunteers were tested for HIV infection with a fourth-generation HIV test (HIV Combi test, Roche Diagnostics) consistent with the United Kingdom National Guidelines on HIV testing (http://www.bashh.org/documents/63/63.pdf). At the final study visit, HIV testing was performed using the HIV Combi test. Positive samples were further tested using Vidas HIV Duo (bioMérieux SA, Marcy l’Etoile, France), Detect HIV, and HIV-1 & -2 Bispot. Samples positive by any of these three tests were further tested by viral RNA PCR to differentiate between vaccine-induced antibodies and HIV infection.

ECG. All ECGs performed during the trial were transmitted electronically to the St. Louis University Core ECG Laboratory (St. Louis, MO) for interpretation.

IFN-γ ELISPOT assay. Cellular immunogenicity was assessed by gamma interferon (IFN-γ) enzyme-linked immunosorbent spot (ELISPOT) assay prior to vaccination, at 4, 8, and 24 weeks over the period of the three DNA vaccinations, and at 2 and 13 weeks after the MVA vaccination in group A. For group B, responses were assessed prior to vaccination, at 2 weeks after each MVA vaccination, and at 13 weeks after the last MVA vaccination. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient separation from heparinized whole blood and were used fresh in a validated IFN-γ ELISPOT assay. For all other assays, PBMC were frozen in 90% fetal bovine serum (FBS) and 10% dimethyl sulfoxide (DMSO) using a Kryo 560-16 rate controlled freezer (Planer, Sunbury-On-Thames, United Kingdom) and stored in vapor phase liquid nitrogen. The PBMC for the IFN-γ ELISPOT assay were plated at 2 × 10⁵ viable cells per well in quadruplicate with peptides at 1.5 μg/ml, representing the vaccine inserts, as described previously (39–41). Peptides were synthesized as 15-mers overlapping by 11 amino acids with ~90% purity by high-pressure liquid chromatography (HPLC) (AnaSpec Inc., Fremont, CA). Six Advax peptide pools containing the sequences of

| Table 1 Study schema | Vaccine given at wk: |
|---------------------|---------------------|
| **Group** | **Vaccine dosage** | 0 | 4 | 8 | 26 |
| A | 3 DNA + rMVA | Advax | Advax | Advax | TBC-M4 |
| B | 3 rMVA | TBC-M4 | TBC-M4 | None | TBC-M4 |

* Twelve vaccine and 4 placebo recipients per group.

* Vaccines: recombinant MVA (rMVA), TBC-M4 at 5 × 10⁷ PFU/dose given intramuscularly; DNA, Advax HIV-1 DNA at 4 mg/dose given intramuscularly by needle-free injection using Biojector 2000.
Gag, Env (2 pools), Pol-RT (2 pools), and Nef-Tat were used. Seven TBC-M4 peptide pools containing the sequences of Gag, Env (2 pools), Pol-RT (2 pools), Nef, and Tat-Rev were used (see Table S1 in the supplemental material). Averages of 129 and 86 samples for Advax and TBC-M4, respectively, were used for assessing the 97.5th percentile cutoff for the ELISPOT assay. These samples were from the baselines from the current trial and other IAVI trials where these peptides were used (16, 37, 39). In group A, cellular immunogenicity was assessed against the peptides for both constructs at enrollment and also after the administration of MVA boost, whereas samples of group B volunteers were assessed using TBC-M4 matched peptides only.

To determine the frequency of positive responses, the Advax Nef-Tat pool (73 amino acids) and the combined TBC-M4 Nef and Tat-Rev pools (93 amino acids) were considered equivalent; likewise responses to each of the 2 Env and 2 Pol-RT pools matching the Advax and TBC-M4 inserts were considered a “hit” for determination of a positive responder. A peptide pool consisting of 9- or 10-mer peptides representing cytomegalovirus, Epstein-Barr virus, and influenza virus (CEF) was used at 1.5 μg/ml, phytohemagglutinin (PHA) was used at 10 μg/ml, and a mock stimulus (DMSO-medium) was used as previously described (40, 42).

Spot-forming cells (SCF) were counted using an automated AID ELISPOT reader (Autoimmun Diagnostika, Strassberg, Germany). The number of SFC/106 PBMC had to satisfy the following criteria. (i) The average number of background-subtracted spots in a single pool had to be more than the specified cutoff of 38 SFC/ml. The cutoffs were derived from the distribution of prevaccination and placebo responses for the vaccine matched peptide pools. (ii) For each pool, if the number of replicates was greater than one, the coefficient of variation (standard deviation/mean) between replicates should be <70%. (iii) The mean count must be >4 times mean background. (iv) The mean background must be <55 SFC/106 PBMC. Assays with mean background >55 SFC/106 PBMC were considered failures. For any subject, if prevaccination ELISPOT responses had a value greater than 38 SFC/106 PBMC, all subsequent responses to that peptide pool in that individual were considered cross-reactive and were not included in the frequency calculations.

ICS and multiparameter flow cytometry. In order to further characterize T-cell responses, ELISPOT-positive samples at 2 weeks after the last vaccination along with matched baseline samples were tested for phenotype and cytokine secretion using a qualified polychromatic flow cytometry assay as described previously (39). Cryopreserved PBMC were thawed and rested overnight, and PBMC were resuspended at 1 × 108 PBMC in 200 μl of RPMI medium supplemented with 10% FBS and coinoculated with 1.5 μg/ml of HIV insert matched peptide pools (ditto ELISPOT) or 1 μg/ml staphylococcal enterotoxin B (SEB) (Sigma-Aldrich, Poole, Dorset, United Kingdom), CD107 phycocerythrin (PE)-Cy5 (Becton, Dickinson, Oxford, United Kingdom), brefeldin A (Sigma-Aldrich, Poole, Dorset, United Kingdom), and BD Golgistop (Becton, Dickinson, Poole, Dorset, United Kingdom) for 6 h at 37°C. Cells were stained with 50 μl Live/Dead fixable blue dead cell stain kit (Invitrogen, Paisley, United Kingdom), anti-CD4 QD605, anti-CD8 Pacific orange, (Invitrogen, Paisley, United Kingdom), anti-CD27–fluorescein isothiocyanate (anti-CD27–FITC) (Becton, Dickinson, Oxford, United Kingdom), and anti-CD45RO (Beckman Coulter, High Wycombe, United Kingdom) and stained intracellularly with anti-CD3–QD655 (Invitrogen, Paisley, United Kingdom), anti-IFN-γ–PE–Cy7, anti-IFN-β–PE–Cy7, anti-IL-2–PE–Cy7, anti-IL-10–PE–Cy7, anti-IL-4–PE–Cy7, anti-IL-17–PE–Cy7, and anti-IL-22–PE–Cy7 (Becton, Dickinson, Oxford, United Kingdom). At least 500,000 events were acquired on a custom-built BD LSRII cytometer. Data were analyzed using FlowJo (Treestar, Ashland, OR), PESTLE, and SPICE (courtesy of Mario Roederer, Vaccine Research Center, National Institutes of Allergy and Infectious Diseases) software. An intracellular cytokine secretion (ICS) responder was considered positive if the background-subtracted values were twice those of the mock stimulus for each CD4- or CD8-cytokine combination.

VIA. The viral inhibition assay (VIA) was performed as described by Spentouz et al. (43). Briefly, PBMC were thawed, divided into two aliquots, and expanded for 7 days with bispecific CD3/CD8 or CD3/CD4 antibodies and IL-2 to generate target CD4 and effector CD8 T cells, respectively, resulting in expansion and enrichment (>90% of the CD3 T cells in the culture) of the required CD4 or CD8 T-cell population. Separate cultures were established containing target CD4 T cells alone infected with either vaccine clade C matched (97ZA012, 247FV2 an infectious molecular clone) or clade B mismatched (IIIB) HIV-1 isolates at a multiplicity of infection (MOI) of 0.01, and virus-infected target cells were cocultured with autologous CD8 effector T cells from the baseline prevaccination blood draw or from samples drawn at each postvaccination time point to be assessed. To limit variation due to effects of the vaccine regimen on the CD4 target cells, a single population of target CD4 cells was generated for each individual; where available, this cell population was generated from the baseline prevaccination sample. Every 3 to 4 days, half of the culture supernatant was removed and replaced with fresh media and assessed for Gag p24 content using a commercially available enzyme-linked immunosorbent assay (ELISA) (PerkinElmer, United Kingdom). CD8 T-cell-mediated inhibition was expressed as log10 reduction in the p24 content of day 13 CD8 and CD4 T-cell cocultures compared with that of CD4 T cells alone. Viruses used for assessment of inhibition were obtained from the National Institutes of Health AIDS Reference and Reagent Program (Gaithersburg, MD), except for 247FV2, which was kindly provided by George Shaw (University of Pennsylvania, PA).

Cutoffs were defined by the 97.5th percentiles of the baseline VIA responses from baseline samples as estimated using PROC QUANTREG in SAS 9.2 (SAS Institute Inc., Cary, NC). VIA response for each virus was defined as positive if the following three criteria were fulfilled: (i) log10 inhibition is greater than 1.5 for the IIIB, 97ZA012, and 247FV2 viruses, (ii) prevaccination response for the same virus is negative (i.e., not cross-reactive), and (iii) the difference between the postvaccination and prevaccination response is ≥0.6 log10 inhibition.

Anti-HIV-specific antibodies. The HIV-specific humoral immune response was evaluated prior to vaccination and at 2 and 13 weeks following the TBC-M4 boost in group A and the third TBC-M4 vaccination in group B using a commercial ELISA diagnostic kit (Elavia Ac-Ab-Ak 1; Bio-Rad Laboratories, Hercules, CA) containing whole-virus lysate as described previously (16). Antibody responses are expressed as the ELISA corrected absorbance as determined from optical density (OD) values as follows: (absorbance of antigen-positive well) minus (absorbance of antigen-negative well). Samples were defined as positive if the following criteria were met: positive control OD was ≥0.8, negative control OD was <0.3, and test sample OD was ≥0.3.

A customized Luminex assay was used to quantify the relative concentration of total IgG to specific HIV proteins. Briefly, carboxylated microspheres (Luminex, Austin, TX) were coupled with clade C gp120, gp41, gp140, or gp24 protein (all recombinant proteins originating from clade C/ZA.1197MB [GenBank accession no. AY463234]; Immune Technology, New York, NY) by covalent [3-dimethylaminopropyl]carbodiimide (EDC) and NHS (Thermo Fisher Scientific, Waltham, MA) in PBS in accordance with the manufacturer’s instruction. Five thousand microspheres of each conjugated protein in PBS–0.1% bovine serum albumin (BSA) were added to each well of a 96-well filter plate (EMD Millipore, Billerica, MA). Fifty microliters of each vaccine sample at 200 μg/ml purified bulk IgG was then added to individual wells of the 96-well plate and incubated overnight at 4°C with shaking. Microspheres were then washed three times with PBS–Tween. Pan-IgG conjugated with PE detection reagent (Southern BioTech, Birmingham, AL) was added to the wells containing bound vaccine antibody. The 96-well plate was incubated with shaking for 2 h, washed three times, and read on a Bio-Plex 200 system (Luminex, Austin, TX) instrument. Background signal, defined as the mean fluo-
orescence intensity (MFI) of microspheres incubated with detection reagents in the absence of clinical antibody, was subtracted from each sample.

Antivector antibodies. Vaccinia virus binding antibody (VVbAb) titers were analyzed at baseline and at 2 and 12 weeks after the last vaccination. A response to vaccinia virus antigen was considered positive if the titer was $>100$ by an in-house binding antibody ELISA using purified vaccinia WR virus as the coating antigen (V-Bio; St. Louis University, St. Louis, MO) (10). A positive response to vaccination was defined as a baseline titer $<100$ and postvaccination titer $>100$ or baseline titer $<100$ and postvaccination titer $>2$ times baseline titer. For prevaccine (baseline) responses, a titer of $>100$ was considered positive.

HIV neutralization. Sera were tested for neutralizing activity against HIV-1 at Monogram Biosciences, Inc. (South San Francisco, CA) as described elsewhere (44). A panel of five subtype C viruses (98IN022, 93IN905, 98CN009, CZA97012, and MGRM-C-26) and three subtype B viruses (SF162, JRCSF, and NL43) were used. Neutralizing activity is displayed as the percent inhibition of viral replication (luciferase activity) at each antibody dilution compared with an antibody-negative control. Titers were calculated as the reciprocal of the plasma dilution conferring 50% inhibition (IC$_{50}$).

Statistics. Fisher’s exact test (for categorical variables) and the Kruskal-Wallis test (for continuous variables) were used to compare the balance and/or values of baseline characteristics between the study groups. All safety and immunogenicity comparisons were made using Fisher’s exact, two-tailed tests of the proportions of volunteers with an endpoint, unless otherwise stated. The safety comparisons were based on the maximum severity per volunteer. All tests are two tailed; statistical significance is defined as a $P$ value of $<0.05$. Analyses were performed using SAS version 9.2 (SAS Institute Inc., Cary, NC).

RESULTS

Participant flow, recruitment, and demographics. Fifty-six volunteers were screened for this study, of whom 32 were enrolled as shown in the Consort diagram (Fig. 1). All vaccinations were administered to all but one volunteer in group A, who was excluded early after the first study vaccination by the investigator because of inability to comply. There were no study terminations or discontinuations of vaccination in any volunteer due to vaccine-related adverse events.

Volunteers were screened between 14 to 21 days before the first study vaccination and were followed for 6 months after the last vaccination. Enrollment began in November 2008 and was completed in April 2009, with clinical follow-up completed in April 2010.

Baseline demographic and clinical characteristics for all trial participants are listed in Table 2. Female volunteers predominated in group A (11/16, 68.8%), whereas male volunteers predominated in group B (11/16, 68.8%). Mean ages and ethnicities were similar in both groups.

Safety and reactogenicity. Both vaccines and regimens were well tolerated. No serious adverse events (SAEs) or grade 3 events were reported. There were 68 nonserious unsolicited adverse events (AEs) reported by 28 volunteers during the course of the trial, of which four events were reported as probably or definitely related to study vaccination in three volunteers. One volunteer (group A, vaccine recipient) experienced finger coldness on the left hand and paraesthesia of the arm/hand on the day of vaccina-
tion, assessed as grade 1 in severity, which resolved on the same day (day of vaccination). Two additional volunteers (group A, vaccine recipients) experienced a subcutaneous nodule at the vaccination site, also assessed as grade 1 in severity.

The percentages of volunteers who experienced local and systemic reactogenicity in groups A and B are presented in Fig. S1 and S2 in the supplemental material. Maximum local reactogenicity for both groups was grade 1, with the most frequently reported events being pain and tenderness (both vaccine and placebo groups) followed by erythema (vaccine groups only). There was no apparent increase in local reactogenicity in either group A or B with subsequent vaccinations. Maximum systemic reactogenicity for both groups was grade 2 and was reported only in vaccine recipients. Headache was the most frequently reported event, followed by nausea, malaise, tiredness, myalgia, and chills. The difference in proportions of volunteers in groups A and B with clinical assessments of systemic reactogenicity that were grade 1 or grade 2 was not statistically significant ($P = 0.36$), with the most frequently reported event assessed as grade 1 in severity.

All troponin-I results were within the institutional reference range except for one result that was slightly outside the range and not considered to be clinically significant. Ninety-six ECG tests were performed, with 35 (36%) reported as normal and 61 (64%) as not normal. Twenty-one (87.5%) of the 24 volunteers who received the study vaccine(s) and all (8) volunteers who received the placebo had non-clinically significant ECG abnormalities reported, which were noted at one or more of the three prespecified testing time points (i.e., at screening, following all study vaccinations, and at the final study visit). Abnormalities included sinus arrhythmia (51/61; 84%), nonspecific intraventricular conduction delay (2 volunteers), right bundle branch block (1 volunteer), and early repolarization changes (2 volunteers) and did not affect study vaccination/conduct.

There were no reports of intercurrent HIV infection or pregnancies during the trial. At the final study visit, 10 of 11 (90.9%) volunteers in group A were noted with vaccine-induced seropositivity (VISP), testing positive on one HIV ELISA (Vidas HIV Duo Quick). Similarly, 100% (12/12) of volunteers in group B were noted with VISP at the final study visit, also testing positive on one HIV ELISA (Vidas HIV Duo Quick).

### TABLE 2 Baseline demographics

| Parameter | Value for: | Group A | Group B | Total |
|-----------|------------|---------|---------|-------|
| No. of:   |            |         |         |       |
| Males     | 5          | 11      | 16      |
| Females   | 11         | 5       | 16      |
| Age (yr)  |            |         |         |       |
| Mean, vaccine group | 36.0 | 34.3 | 35.2 |
| Mean, placebo group | 40.8 | 40.0 |       |
| Range, vaccine group | 20–48 | 23–49 | 20–49 |
| Range, placebo group | 29–50 | 29–50 |       |
| Race/ethnicity |         |         |         |       |
| Asian     | 0          | 1       | 1       |
| Black     | 0          | 1       | 1       |
| White     | 15         | 14      | 19      |
| White, black" | 1       | 0       | 1       |

a White, black, one individual checked both the “White” and “Black” boxes.

### TABLE 3 Frequency of positive IFN-γ ELISPOT responses with fresh PBMC postvaccination

| Overall response rate in: | Group A tested with: | Group B tested with TBC-M4 peptide pools | Placebo group tested with any peptide pool |
|---------------------------|----------------------|-----------------------------------------|------------------------------------------|
| Time of assay$^a$ | n | % | n | % | n | % |
| Post-2nd | 0/10 | 0 | NS | NS | 6/12 | 50 | 1/7 | 14.3 |
| Post-3rd | 1/10 | 10 | NS | NS | 7/11 | 63.6 | 3/7 | 42.9 |
| Post-4th | NS | NS | 9/9 | 100 | NA | NA | 1/4 | 25 |

$^a$ Post-2nd, after the second vaccination.

*b, number of positive responders/number of subjects after each vaccination; NS, not shown; NA, not applicable.

$^c$ At the second and third vaccination visits, subjects were tested with Advax and TBC-M4 peptide pools in group A, but only Advax peptide pools are included. Only TBC-M4 peptide pools were tested in group B.

$^d$ At the fourth vaccination visits for group A, subjects were tested with Advax and TBC-M4 peptide pools, but only Advax peptide pools are included.

### IFN-γ ELISPOT responses

The IFN-γ ELISPOT was the primary assay for assessing cellular responses among groups in this study and was performed longitudinally. Group A responses were assessed at the following time points: prevaccination, at 2 and 4 weeks after the second DNA vaccination, prior to the MVA at 26 weeks, and at 2 and 13 weeks after the MVA boost. Group B responses were assessed at the following time points: prevaccination, 2 weeks after the 2nd MVA vaccination, and at 2 and 13 weeks after the 3rd MVA vaccination (boost). A total of 172 fresh PBMC samples were assessed in the ELISPOT, with 17 failed samples that did not meet the predefined acceptability criteria. The viability of the fresh PBMC was always exceeded 95%, and all samples were processed within 3 h of blood draw. The average ELISPOT magnitudes for mock, PHA, and CEF stimulations for 155 specimens were 10, 1,053, and 390 SFC/million PBMC, with standard deviations (SD) of 9, 630, and 598 SFC/million PBMC, respectively.

IFN-γ ELISPOT responses to any of the Advax matched peptide pools were detected in only 1 individual out of 10 (10%) in group A following Advax immunizations; this rate increased to 9/9 individuals (100%) responding to any TBC-M4 matched peptide pools after the MVA boost. In group B, IFN-γ ELISPOT responses to any TBC-M4 matched peptide pools were detected in 6/12 (50%) and 7/11 (64%) individuals after the second and third MVA vaccinations, respectively (Table 3). At 2 weeks after the last MVA vaccination, the response rates of 9/9 (100%) volunteers in group A and 7/11 (64%) in group B were not significantly different. Sporadic false-positive responses to both the Advax and TBC-M4 matched peptide pools were seen in placebo recipients, with an overall false-positive rate of 12/647 baseline and placebo samples across the 13 peptide pools (1.8%). There was a significant difference ($P = 0.014$, Fisher’s exact two-tailed test) in the response rate between the vaccinees (9/9) and placebo recipients (1/4) at 2 weeks after the second vaccination in group A when using Advax peptides. The differences in the response rates between the vaccinees and placebo recipients at 2 weeks after the second vaccination in group A and B when using TBC-M4 peptide pools were not significant.
In group A, where two sets of peptides (Advax and TBC-M4) were used for detection of ELISPOT responses, the magnitudes of the responses to the two sets of Env, Pol, and Gag peptides were similar at 2 weeks after the last vaccination (Fig. 2). The geometric means of SFC/million PBMC were 215, 67, and 61 for Advax and 196, 57, and 72 for the TBC-M4 peptides for Env, Pol, and Gag, respectively, for all samples postvaccination. In group B only the TBC-M4 peptides were used; after the last vaccination the geometric means of SFC/million PBMC for Env, Gag, and Pol were 57, 14, and 35, respectively, for all samples postvaccination. The geometric mean responses to any antigen (combining Env, Pol, and Gag) were 365 and 348 for the Advax and TBC-M4 peptides in group A, respectively, and 128 for the TBC-M4 peptides in group B. Using the normal approximation of the nonparametric Wilcoxon two-sample test at week 28, there were significant differences between group A and B ELISPOT geometric mean magnitudes using the TBC-M4 matched peptides for each of the Env, Gag, and Pol peptide pools and for the summed magnitude of Env plus Gag plus Pol peptides. In group A after the last vaccination, the ELISPOT magnitude and response rates for Env were greater than those for Gag or Pol. In group B the magnitudes and response rates were lower and there was no apparent hierarchy of responses to Env, Gag, and Pol. At the peak of the response (2 weeks after the last vaccine), in group A 3/9 (33%) vaccinees responded to both the Advax Nef/Tat and to the TBC-M4 Nef peptide pools. There was one responder to the Rev pool in groups A and B. No individuals receiving placebos responded to the Advax Nef/Tat or to the TBC-M4 Nef or Rev peptide pools at 2 weeks after the last vaccine.

Finally, the breadth of response to the 7 different peptide pools was assessed using the ELISPOT assay at 2 weeks after the last vaccination with any of the 7 TBC-M4 peptide pools. In group A, 9 individuals had an ELISPOT response; 6 pools were recognized by one individual and 4 by another individual, 2 had responses to 3 pools, and 3 individuals had responses to 2 pools, with the remaining 2 having a response to one pool. In group B, there were 7 responders: 2 individuals recognized 2 pools and the remaining 5 recognized one pool each. In the placebo group, there were 2 individuals who recognized peptide pools; one recognized 1 pool, and the other recognized 2 pools. Overall, a medians of 2 pools, 1 pool, and 0 pools were seen in groups A and B and the placebo group, respectively (Fig. 3).

Viral inhibition assay. VIA was performed against three HIV isolates: 247FV2 (clade C), 97ZA012 (clade C), and IIIB (clade B). In group A, 4 individuals had VIA activity against the clade C isolate (247FV2), with log_{10} reductions in p24 of 4.8, 1.7, 2.8, and 2.8 at 2 weeks after all 3 Advax and TBC-M4 vaccinations had been administered. In group A, two individuals had VIA activity against clade B (IIIB), both with a log_{10} reduction in p24 of 3.2. None in group A had responses to the clade C isolate (97ZA012). The positive VIA responses were confirmed in 2 separate experiments for five of the six samples, with equivalent activity detected in both experiments (data not shown). VIA activity in the 6th positive
sample could not be repeated due to insufficient sample availability; all other repeated samples had negative VIA responses. Overall, five individuals in group A had VIA responses, one individual to two viruses and four individuals to one virus. Three individuals in group A had responses at baseline to the 247FV2 virus (1.65 log_{10}) and IIIB (1.6 log_{10}) just above the cutoff; however, by the criteria in Materials and Methods they are nonresponders. One was a placebo recipient and two were vaccinees. There were no positive VIA responses in vaccinees in group B to any of the three viruses. Overall there was a significant difference in the VIA response rate of 5/11 in group A and 0/12 in group B (P = 0.0137, Fisher’s exact two tail).

**Intracellular cytokine flow cytometry.** Of the 32 volunteers enrolled in the study, samples for ICS analysis were taken from 14 volunteers at 2 weeks after the last administration and their corresponding baseline samples. Specimens were chosen based on the presence of an ELISPOT response and assessed together with several random placebo samples. Six vaccinee and 2 placebo samples were assessed in group A, and 5 vaccinee samples and 1 placebo sample were assessed in group B. Samples that had higher-magnitude responses at 2 weeks postvaccine compared to those at baseline were all in group A. The group A sample responses were predominantly CD4, and the cells secreted IL-2, IFN-γ, and TNF-α mainly in response to Env stimulation. The ranges of CD4^+ Env-specific responses were 0.032 to 0.093% for CD4^+ IFN-γ^+ T cells, 0.036 to 0.093% for CD4^+ IL-2^+ T cells, and 0.048 to 0.092% for CD4^+ TNF-α^+ T cells. All samples included in the ICS analysis had responses to the positive control (SEB). Figure S3 in the supplemental material shows one individual’s TBC-M4 Env pool 1-specific ICS response at 2 weeks postvaccination; the total CD4 IFN-γ response was 0.047%, and the CD8 response was 0.015%. The SEB responses in this volunteer were 4.9% and 10% for CD4 and CD8, respectively. At baseline this individual had a CD4^+ ICS response to this peptide pool of less than 0.033%; the profile shows that most cells were of the central or memory effector phenotype.

**ELISA anti-vaccinia virus binding antibodies.** At baseline there were 3/11 (27.3%), 3/12 (25%), and 3/8 (37.5%) individuals with anti-vaccinia virus antibodies in groups A and B and the placebo group, respectively. As shown in Fig. 4 for vaccine recipients, at 2 weeks after the last vaccination, the response rates were 100% in group B and 18.2% in group A (Fisher’s exact two-tailed test; P < 0.0001). Similarly, at the 9-month visit, 13 weeks after the last vaccination, the response rates were 100% in group B and 27.3% in group A (P < 0.0003). The median anti-vaccinia virus titers at 2 weeks after the last vaccination were 1,855 in group B and <100 in group A (Wilcoxon two-sample test using the two-tailed t approximation; P = 0.0068), and at the 9-month visit the median titers were 745 and <100 in groups B and A, respectively (P = 0.0220).

**HIV-specific antibody responses.** There were no positive HIV-specific antibody responses detected in placebo samples or at baseline for any volunteer using the Elavia assay. As shown in Fig. 5, at 2 weeks after the last vaccination, the response rates were 100% in group B and 9.1% in group A (Fisher’s exact two-tailed test; P < 0.0001). Similarly at the 9-month visit, 13 weeks after the last vaccination, the response rates were 100% in group B and 18.2% in group A (P = 0.0002). The median anti-HIV antibody OD values at 2 weeks after the last vaccination were 2.814 in group B and 0.068 in group A (P = 0.0006), and at the 9-month visit the median titers were 2.3 and 0.151 in groups B and A, respectively (P = 0.0007). Statistical analysis was by the Wilcoxon two-sample test using the two-tailed t approximation.

Assessment of antibodies to rgp41Env, rgp140 Env, p24 Gag, and rgp120 Env confirmed that individuals in group B had significantly higher relative titers than those in group A or the placebo group (Fig. 6). In group B, the geometric mean antibody titers to rgp41, rgp140, p24, and rgp120 were 4,886, 3,223, 11,643, and 3,344, respectively (Fig. 6A to D, respectively). Thus, antibodies to
Gag p24 were much higher than those to any of the Env components.

**HIV neutralization.** In group A, 1 of 8 placebo recipients (13%) and 1/11 (9%) vaccinees had a positive HIV neutralizing response (both to the MGRM-C-26 virus). In group B vaccinee samples, there were 5/12 (42%) responses: one volunteer had weakly positive HIV neutralizing antibody responses to 8 HIVs, and another 4 volunteers also had positive responses to the SF162 virus. The positive HIV neutralizing titers were all less than 100 at IC_{50} (data not shown).

**DISCUSSION**

This study shows that the administration of DNA plus MVA (group A) in a prime-boost regimen or MVA alone (group B) was safe and moderately immunogenic in humans. Both vaccination regimens demonstrated an acceptable safety profile and were well

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**FIG 5** Vaccine-induced anti-HIV antibodies (Bio-Rad Laboratories; Elavia) and responder rate (%) by study visit and dose group. White and black arrows indicate vaccinations with Advax (4 mg DNA) and TBC-M4 (5 × 10^7 PFU MVA), respectively. Black and white dots are positive and negative titers, respectively.

**FIG 6** Vaccine-induced antibodies to rgp41 (A), rgp140 (B), p24 (C), and rgp120 (D). The relative concentrations of total IgG to specific HIV proteins were assessed at 4 weeks after the last vaccination using a customized Luminex assay. Statistical differences using one-way analysis of variance (ANOVA) are indicated: ***, P < 0.0001; **, P < 0.001.
tolerated. All local reactogenicity was mild in severity, and no systemic reactogenicity was greater than moderate, with no statistical difference noted between vaccine and placebo recipients. Un- solicited adverse events were mild or moderate in severity, with no serious adverse events reported. Biojector administration of the DNA vaccine was well tolerated. ECGs taken at screening, following all study vaccinations, and at the final study visit were notable for non-clinically significant findings, mainly sinus arrhythmia, which were seen at all time points.

Only one individual out of 10 had an ELISPOT response after the three Advax immunizations, which is similar to the result seen after administration of Advax alone in previous trials (37, 39). Limited potency of DNA alone or as part of a prime-boost strategy has been seen in other trials (15, 21, 24, 45). The DNA backbone, dose, number of administrations, insert, and method of delivery are all variables associated with effectiveness of the DNA (8, 46, 47). Some studies have shown that DNA alone is immunogenic in humans, albeit with low-magnitude responses as measured by ELISPOT and/or ICS (26, 48, 49). Use of Biojector does not seem to enhance immune responses to DNA in either humans or non-human primates, though few studies have directly compared standard intramuscular administration of DNA to Biojector in humans (49–51). In contrast, 7 of 11 individuals had an ELISPOT response to 3 doses of TBC-M4, though the mean magnitude of the response was modest. The response rate and magnitude are similar to those seen in other MVA- or NYVAC-only trials (12, 16, 24).

For the combined vaccine regimen, in spite of the absence of a detectable priming of the immune response, group A had T-cell responses superior to those in group B. In group A, the ELISPOT magnitude was greater than that in group B, and only in group A was CD8 T-cell-mediated VIA activity detected. In addition there was a trend toward greater magnitude of ICS and greater breadth across proteins in group A than B. This hidden priming effect, where the immune response quality is enhanced by the DNA prime, has been seen in trials where adenoviral or poxvirus vectors are used as a boost (18, 23, 24, 26, 45, 48). The immune response quality and quantity appear to be enhanced by the “hidden” DNA prime and manifests itself by increased polyfunctionality of the T cells and increased numbers of terminally differentiated T cells with a cytotoxic effector potential (19, 52). In spite of the small numbers of volunteers in this trial, 5 out of 11 individuals had VIA activity in group A compared to none out of 12 in group B. VIA activity has not been detected after DNA, MVA, or canarypox virus and protein boost regimens but is generally potent after administration of adenovirus vectors (43, 53). The VIA data indicate that the quality of the immune response may be enhanced by the DNA prime.

In contrast to the cellular responses, the magnitude and frequency of HIV-specific antibody responses were dramatically better in group B than in group A at the peak of the response after all vaccinations had been administered. Although we did not look at the antibody response after 3 doses of Advax, we know from previous studies that weak antibodies are detected after Advax delivered intramuscularly or by electroporation (37, 39). In the study presented here, volunteers in group A received only one administration of MVA compared to three doses of MVA in group B. Nonetheless, it did not appear that Advax enhanced the antibody response in group A. In a previous study, after immunization with TBC-M4 most individuals who were vaccinia virus naïve seroconverted after the first or second immunization, as indicated by this same whole-virus ELISA (16). Although the antibody responses were robust in group B, only weak neutralizing antibodies were induced. The induction of potent neutralizing antibodies has not been achievable to date by administration of any vaccine regimen in humans or macaques although great strides are being made (54–57). Poxviruses administered alone or with a protein boost lead to the induction of high-titer ELISA antibodies, but these antibodies are weakly neutralizing to tier 1 viruses. Notably, the RV144 and associated vaccines elicited mostly tier 1 antibodies (58). In contrast the administration of canarypox virus and protein in the RV144 study elicited nonneutralizing V1V2-specific antibodies with the potential ability to protect from infection with HIV (59). We did not assess the ability to induce nonneutralizing antibodies in this vaccine trial; however, there are indications that poxvirus and adenovirus vaccines can elicit antibodies with V1V2 binding specificity similar to that seen in the RV144 trial (60).

At 2 weeks after three immunizations with DNA and a single MVA immunization in group A, three individuals who had detectable VVbAb at baseline (27.3%) had a boost in ELISA titers but there were no new seroconverters. In group B three individuals who had detectable VVbAb at baseline (25%) had a boost in ELISA titers and the other 9 individuals seroconverted with high-titer VVbAb after 3 immunizations with MVA at 2 weeks after the last vaccination. Because of the small sample size, we were not able to determine whether the preexisting VVbAb had an impact on the HIV-specific responses postvaccination. The antivector antibody responses are similar to those seen in other DNA-MVA or MVA-alone studies. In these studies, there was no noticeable impact of preexisting VVbAb or those generated during the course of the vaccinations on T-cell responses (12, 16, 17, 61). However, all these data are from small phase I trials, which may not have had the power to detect the impact of VVbAb on T-cell responses. In studies with DNA prime and adenovirus vector boost, in spite of very high levels of anti-adenovirus type 5 (anti-Ad5) neutralizing antibodies, robust T-cell responses were still generated, and there may be subtle advantages of a DNA prime followed by a single Ad5 boost compared to two Ad5 administrations (50).

Overall this study showed that the Advax and TBC-M4 vaccines were safe and modestly immunogenic. There was some indication that the DNA enhanced T-cell priming, leading to superior VIA responses and increased breadth in the DNA plus MVA compared to MVA-alone group. Antibody titers were robust after administration of three doses of MVA, but these antibodies were weakly neutralizing.

ACKNOWLEDGMENTS

We are very grateful to David Ho and Sandhya Vasan from the Aaron Diamond AIDS Research Center (New York, NY) for providing the Advax DNA vaccine and necessary support for this clinical trial. We also thank the IAVI regulatory affairs team for all aspects of vaccine preparedness. We acknowledge the members of the IAVI Human Immunology Laboratory, especially Gwynn Stevens and Paramesh Chetty, for technical and logistic support throughout the trial. Not least we thank the volunteers who participated in this trial.

This study was sponsored by the International AIDS Vaccine Initiative and funded by its donors, including the United States Agency for International Development (USAID Cooperative Agreement Number GPO-A-00-06-00006-00), the Governments of Canada, Denmark, Ireland, The Netherlands, Norway, Sweden, and the United Kingdom, the Basque Autonomous Government, the European Union, and the Bill & Melinda
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