MAJOR ARTICLE

High-Dose Recombinant Canarypox Vaccine Expressing HIV-1 Protein, in Seronegative Human Subjects

Paul A. Goepfert,[1] Helen Horton,[2] M. Juliana McElrath,[2] Sanjay Gurunathan,[3] Guido Ferrari,[4] Georgia D. Tomaras,[4] David C. Montefiori,[4] Mary Allen,[5] Ya-Lin Chiu,[2] Paul Spearman,[7] Jonathan D. Fuchs,[8] Beryl A. Koblin,[9] William A. Blattner,[6] Sharon Frey,[11] Michael C. Keefer,[10] Lindsey R. Baden,[12] Lawrence Corey,[2] and the NIAID HIV Vaccine Trials Network

[1]Department of Medicine, University of Alabama at Birmingham, Birmingham; [2]HIV Vaccine Trials Network and Fred Hutchinson Cancer Research Center, Seattle, Washington; [3]Aventis, Swiftwater, Pennsylvania; [4]Central Immunology Laboratory, Duke University Medical Center, Durham, North Carolina; [5]Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, and [6]Institute of Human Virology, University of Maryland at Baltimore, Baltimore; [7]Vanderbilt University School of Medicine, Nashville, Tennessee; [8]San Francisco Department of Public Health, San Francisco, California; [9]The New York Blood Center, New York, and [10]University of Rochester Medical Center, Rochester, New York; [11]Saint Louis University School of Medicine, Saint Louis, Missouri; [12]Division of Infectious Disease, Brigham and Women's Hospital, Boston, Massachusetts

Background. In clinical trials, canarypox ALVAC–human immunodeficiency virus (HIV) vaccines have been shown to elicit human HIV–specific cytotoxic T lymphocyte (CTL) responses in some but not all healthy uninfected adults.

Methods. A clinical trial was conducted to examine whether the vaccine vCP1452 would elicit a greater HIV-specific CTL response when given at a dose of $10^{8.0}$ $TCID_{50}$ (60 participants) than when given at the regular dose, $10^{7.26}$ $TCID_{50}$ (40 participants); as a control, a placebo vaccine preparation also was administered (10 participants).

Results. Two weeks after the last vaccination in a series, HIV-specific CTL responses were not significantly different when measured by either chromium-release assay (8% and 16% in the high- and regular-dose recipients, respectively) or interferon-γ ELISpot assay (8% and 15% in the high- and regular-dose recipients, respectively); moreover, recipients of the higher dose had greater local and systemic reactions ($P < .001$).

Conclusions. High reactogenicity associated with an increased dose of vCP1452 negates the need for further evaluation of this strategy to boost the frequency of HIV-specific CTL response in seronegative human subjects. Development of highly immunogenic canarypox vectors requires further work to optimize vector and insert design, as well as novel ways to increase dosage and to reduce reactogenicity.

With >5 million new HIV-1 infections occurring annually, there is an urgent need to develop a preventive vaccine. Ideally, such a vaccine would elicit both memory cytotoxic T lymphocyte (CTL) responses and HIV-specific neutralizing antibodies. Unfortunately, the currently available envelope-subunit immunogens have not been found to elicit potent HIV-specific neutralizing antibodies against primary isolate strains [1–4], and vaccines that elicit only neutralizing antibodies against T-cell line–adapted HIV strains are not protective against infection or disease [5].

It is clear that HIV-specific CTL response plays an important role in the control of HIV replication; in chronically infected rhesus macaque monkeys, depletion of CD8 T cells causes significant increases in plasma levels of simian immunodeficiency virus (SIV) [6, 7]. Moreover, several studies of monkeys challenged with SIV/HIV have demonstrated that vaccine-induced

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Reprints or correspondence: Dr. Paul A. Goepfert, University of Alabama at Birmingham, Dept. of Medicine, Div. of Infectious Diseases, 908 20th St. S, CCB 328, Birmingham, AL 35294-2050 (paulg@uab.edu).

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Table 1. Study schema.

Group (no. of participants)	ALVAC (vCP1452) dose	Injections, no.[a]	Schedule			
			Month 0	Month 1	Month 3	Month 6
A (60)	$10^{8.0}$ $TCID_{50}$	2[b]	vCP1452	vCP1452	vCP1452	vCP1452
B (40)	$10^{7.26}$ $TCID_{50}$	2[b]	vCP1452	vCP1452	vCP1452	vCP1452
C (10)	…	2[c]	Placebo	Placebo	Placebo	Placebo

[a] One injection administered intramuscularly into each anterior thigh (vastus lateralis).
[b] Each injection contained 2.4 mL of ALVAC-HIV (vCP1452) reconstituted in either sterile water for injection (group A) or 0.9% NaCl (group B).
[c] Half of group C were randomized to receive PLACEBO-ALVAC reconstituted with 0.9% NaCl, whereas the other half was randomized to receive PLACEBO-ALVAC reconstituted in sterile water for injection.

CTL response can prevent disease progression in challenged monkeys [8–10]. In humans, one strategy to elicit HIV-specific CTL response employs a live attenuated viral vector that encodes HIV-specific gene products. Recombinant vaccinia viruses have been used to successfully deliver HIV proteins to the host immune system [11–13]; however, these vectors may cause severe illness when given to immunocompromised persons [14], and T cell responses are diminished in vaccinia-experienced subjects [15]. In contrast to vaccinia virus, canarypox is host-range restricted [15–17]; it fails to replicate in mammalian cells and does not produce infectious virus. Canarypox-based vectors have been associated with an excellent safety profile, in both HIV-infected and -uninfected adults. Because of their large size, canarypox vectors can encode the majority of HIV-specific proteins, delivering them to antigen-presenting cells and producing subsequent elicitation of CTL response.

In previous studies, recombinant canarypox vectors have been shown to be immunogenic in uninfected healthy human recipients, eliciting HIV-specific CTL responses in up to 35% of recipients, when measured at 2 weeks after final vaccination [3, 18, 19]. In an effort to make the vaccine more immunogenic, a recombinant, ALVAC-HIV (vCP1452), was engineered. This construct differs from previous HIV recombinant canarypox products in that it encodes 2 vaccinia-virus genes, which have been shown to inhibit apoptosis of the infected cells [20]. HIV Vaccine Trials Network Protocol 039 sought to determine whether high doses of vCP1452 would elicit an increased HIV-specific CTL response, compared with the response elicited by the regular dose.

SUBJECTS, MATERIALS, AND METHODS

Participants and Study Design

The present study was a multicenter, double-blind, randomized trial conducted at 9 HIV-vaccine trial units. The study protocol was reviewed and approved by the institutional review boards at each site; and informed consent was obtained from each volunteer, in accordance with the guidelines of the US Department of Health and Human Services and of the institution at each site. Healthy participants at low risk of acquisition of HIV infection were recruited as described elsewhere [22]. After providing informed consent, participants were screened for the study. All groups received 2 intramuscular injections, 1 in each thigh (vastus lateralis), at months 0, 1, 3, and 6 (table 1). On days 1 and 2 after each injection, participants self-reported to the clinic any systemic and local reactions that had occurred. The severity of a reaction was defined as follows: (1) mild—transient or minimal symptoms; (2) moderate—notable symptoms requiring modification of activity; and (3) severe—incapacitating symptoms requiring bed rest and/or resulting in loss of work or social interaction. The clinical and laboratory safety data were reviewed weekly during the vaccination period, and data on adverse events (AEs) were collected for 18 months [3, 18, 23, 24]. At every visit to the clinics, risk-reduction counseling was provided, and questions regarding behavioral risk were asked. Immune-assay time points were chosen, in part, on the basis of earlier kinetic studies using the mouse [25] and to compare the results of the present study with those of earlier trials using HIV-recombinant canarypox.

Vaccine

Recombinant canarypox ALVAC-HIV (vCP1452) is a preparation of (1) a modified recombinant canarypox virus expressing the products of the HIV-1 *env, gag,* and *pol* genes, encoding protease, and (2) a synthetic polypeptide encompassing several known human CTL epitopes from the *nef* and *pol* gene products [21]; it was generated by inserting the vector-modifying sequences encoding E3L and K3L into the C6 site of recombinant ALVAC-HIV (vCP1433). The current manufacturing process, using pathogen-free chicken embryo–fibroblast cell lines, yields a maximum dose of $10^{7.2}$–$10^{7.6}$ pfu/mL of vaccine.

The regular-dose preparation of vCP1452, $10^{7.26}$ $TCID_{50}$, was reconstituted in 4.8 mL of 0.9% NaCl and was divided evenly between 2 syringes; the high-dose preparation of vCP1452, $10^{8.0}$ $TCID_{50}$, contained exactly 6 doses of the regular-dose preparation and was reconstituted in 4.8 mL of sterile water for injection (SWFI), and each dose was divided evenly between 2 syringes. The high-dose preparation was chosen on the basis of formulation limitations and single-dose injection volumes

that would be considered clinically acceptable. The placebo preparation contained virus stabilizer and freeze-drying medium that were reconstituted in either SWFI or 0.9% NaCl. At each vaccine-administration time point, 2.4 mL was injected into each thigh.

Immune Assays

Vaccinia constructs. Two sets of autologous stimulator cells for in vitro stimulation (IVS) were infected with (1) recombinant vaccinia virus vP1291, expressing the extrinsic gene inserts HIV-1_{MN} *env* gp120+gp41 transmembrane protein and HIV-1_{LAI} *gag*/protease, and (2) recombinant vaccinia virus vP1558, expressing the HIV-1_{LAI} *nef/pol* epitopes string. Autologous B lymphocyte cell line (BLCL) targets for the CTL assays were infected with recombinant vaccinia viruses vP1170 (Western Reserve parent control), vP1174 (HIV-1_{MN} *env* gp160), vDK1 (HIV-1_{LAI} *gag*), vP1174 (HIV-1_{IIIB} *pol*), or vTFnef (HIV-1_{IIIB} *nef*). All of the recombinant vaccinia constructs were used at a 5:1 MOI. These viruses were provided by Dr. James Tartaglia (Aventis Pasteur) and by the National Institutes of Health AIDS Research and Reference Reagent Program.

CTL responses. $CD8^+$ CTL responses were evaluated after IVS of peripheral-blood mononuclear cells (PBMCs) by recombinant poxvirus–infected autologous PBMC stimulators [26]. Assessments of specific CTL activity were performed 14–18 days after antigen-specific stimulation, by use of autologous ^{51}Cr-labeled target cells infected with the appropriate recombinant vaccinia constructs. Effector-cell populations (i.e., $CD4^+$ or $CD8^+$ T cells) were determined on the basis of depletion by monoclonal antibody–coated magnetic beads (Dynal), as described elsewhere [26]. When vaccinia-infected target cells were used, unlabeled BLCLs infected with the vaccinia control vector served as cold-target competitors to reduce the anti-vaccinia reactivity measured by the CTL assay. Both the percentage of specific lysis and positive results were calculated according to published methods [26]. Not all CTL assays were completed—in some cases because of the failure to establish an immortalized B cell line, in other cases because of the failure of IVS cells to thrive in culture.

Interferon (IFN)–γ ELISpot assay. An IFN-γ ELISpot kit (Becton Dickinson) was used according to the manufacturer's directions. PBMCs were thawed in R10 (RPMI 1640 supplemented with 10% fetal bovine serum, 2 mmol/L L-glutamine, and 25 mmol/L HEPES buffer, 50 μg/mL streptomycin, and 50 U/mL penicillin) containing 50 U/mL Benzonase (Novagen); were washed and rested overnight, before the assay, in R10 at 37°C and in an atmosphere of 5% CO_2; and then were plated at a concentration of 2×10^5 cells/well. Peptides were added to the wells, at a final concentration of 1 μg/mL. Wells containing medium alone served as the negative controls, and wells containing 1 μg/mL phytohemagglutinin (PHA)-P (Murex; Remel) served as the positive controls; negative controls were tested in 6 replicate wells, and peptide antigens and PHA were tested in 3 replicate wells. The next day, the plates were developed according to the manufacturer's instructions, and spots were counted by use of a CTL analyzer and software (version 2.8; CTL Analyzers LLC). Positivity was defined as >55 spot-forming cells (SFCs)/10^6 PBMCs and >4 times the level of the matching negative control.

Binding antibodies. Anti-Gag–binding antibody responses were determined by qualitative and quantitative validated ELISAs. Serum from cryopreserved samples was tested in duplicate in microtiter plates coated with purified p24 Gag (Protein Sciences). For the qualitative ELISA screen of all samples, serum was examined at a dilution of 1:50, to quantify the antibody-positive samples on which quantitative (end point) ELISAs then were performed. A sample was regarded as antibody positive if the difference between the scores of duplicate antigen-containing and non–antigen-containing wells (i.e., the optical density [OD] of the antigen-containing well minus the OD of the non–antigen-containing well) was ⩾0.2. Binding-antibody titers were calculated on the basis of the standardized positive control value, with use of the 50% maximal binding point.

Neutralizing antibodies. Neutralization of HIV-1_{MN} was measured in MT-2 cells, as described elsewhere [4]. Neutralizing-antibody titers are the reciprocal of the serum dilution at which 50% of cells are protected from virus-induced killing as measured by uptake of vital dye (neutral red). Heterologous neutralization was assessed in a luciferase reporter-gene assay in TZM-bl cells, by use of 11 molecularly cloned, clade B pseudoviruses, as described elsewhere [27]. The assay stock of HIV-1_{MN} was generated in H9 cells.

Canarypox antibodies. Microtiter plates were coated, at 37°C for 1 h and at 4°C overnight, with 100 ng/well CPpp antigen, in 0.05 mol/L sodium carbonate buffer (pH 9.6). Plates were then blocked, at 37°C for 1 h, in a solution of 100 μL of PBS (pH 7.1), 0.1% Tween 20, and 1% (wt/vol) bovine serum albumin (PBS-Tween-BSA). All incubated plates were washed 4 times with a solution of PBS (pH 7.1) and 0.1% Tween 20. Serial 2-fold dilutions of the samples in PBS-Tween-BSA, starting at 1:100, were added to the wells, which were then incubated at 37°C for 90 min. After 4 washings, an anti–human IgG peroxidase conjugate (SIGMA) diluted in PBS-Tween-BSA at 1:15,000 was added, and the plates were incubated at 37° C for an additional 90 min. The plates were washed an additional 4 times and were incubated, in the dark for 30 min at room temperature, with a 3,3′,5,5′-tetramethylbenzidine substrate (Tebu BioLaboratories). The reactions were stopped with 100 μL of 1 N HCl (PROLABO). The OD was measured at 450–650 nm, by use of an automatic plate reader (VersaMax; Molecular Devices).

Table 2. Demographic characteristics of trial participants.

	Group, no. (%) of participants			
	A (n = 60)	B (n = 40)	C (n = 10)	Total (n = 110)
Sex				
Male	38 (63)	28 (70)	5 (50)	71 (65)
Female	22 (37)	12 (30)	5 (50)	39 (35)
Sexual preference				
Homosexual	19 (32)	14 (35)	1 (10)	34 (31)
Heterosexual	34 (57)	21 (53)	9 (90)	64 (58)
Bisexual	7 (12)	5 (13)	0 (0)	12 (11)
Race/ethnicity				
White, non-Hispanic	44 (73)	24 (60)	7 (70)	75 (68)
African American, non-Hispanic	10 (17)	7 (18)	1 (10)	18 (16)
Hispanic	4 (7)	3 (8)	2 (20)	9 (8)
Asian/Pacific Islander	1 (2)	3 (8)	0 (0)	4 (4)
Native American/Alaskan Native	0 (0)	0 (0)	0 (0)	0 (0)
Other	0 (0)	1 (3)	0 (0)	1 (1)
Multiracial	1 (2)	2 (5)	0 (0)	3 (3)
Age				
18–20 years	3 (5)	1 (3)	1 (10)	5 (5)
21–30 years	28 (47)	15 (38)	3 (30)	46 (42)
31–40 years	14 (23)	13 (33)	3 (30)	30 (27)
41–50 years	14 (23)	11 (28)	3 (30)	28 (25)
>50 years	1 (2)	0 (0)	0 (0)	1 (1)
Median age, years	30.0	33.0	36.0	31.5
Age range, years	19–55	20–46	20–50	19–55
Vaccination(s) received				
Day 0	60 (100)	40 (100)	10 (100)	110 (100)
Day 28	57 (95)	39 (98)	10 (100)	106 (96)
Day 84	51 (85)	37 (93)	9 (90)	97 (88)
Day 168	51 (85)	36 (90)	9 (90)	96 (87)

Statistical Analysis

Safety assessments (local and systemic reactogenicity rates and rates of AEs) were compared, between groups, by use of a nonparametric Kruskal-Wallis test. Immunogenicity response rates (CTL, ELISpot, and neutralizing antibody) were estimated by use of exact 95% confidence intervals (CIs) and were compared between groups by use of Fisher's exact test. Log titers for antibody results were described in terms of arithmetic and geometric means and were compared by use of nonparametric Wilcoxon rank-sum tests.

RESULTS

Participant Accrual, Demographic Data, and Vaccine Safety

Of the 110 participants enrolled, the majority were white, non-Hispanic males in their 20s, but other groups also were represented (table 2). All 110 participants received the first vaccination, 106 received the second, 97 received the third, and 96 received the fourth; the primary reason that the series of vaccinations was not completed was the occurrence of vaccine-induced local or systemic symptoms, which occurred in 8 of the recipients of the high-dose ($10^{8.0}$ $TCID_{50}$) preparation of vCP1452. Recipients in the youngest age group (age, 18–30 years) were more likely to discontinue subsequent vaccinations (11/51 [22%]), compared with the rest of the cohort (3/59 [5%]) ($P = .02$).

The most common symptoms were pain, tenderness, malaise, and myalgia (figure 1 and Appendix A). The high-dose preparation was associated with a significantly higher frequency and intensity of local and systemic side effects, compared with either the regular-dose preparation or the placebo preparation (figure 1 and Appendix A), and this difference was most evident in the elevation in body temperature: within the first 48 h after vaccination, 78% (47) of those receiving the high-dose preparation had a temperature ⩾100°F, and 50% (30) had a temperature ⩾101°F (Appendix A). Other side effects seen more frequently in recipients of the high-dose preparation than in recipients of either the regular-dose preparation or the placebo preparation included local pain or tenderness, malaise, myalgia, headache, and nausea. Also, compared with those receiving placebo, regular-dose recipients experienced more local pain or

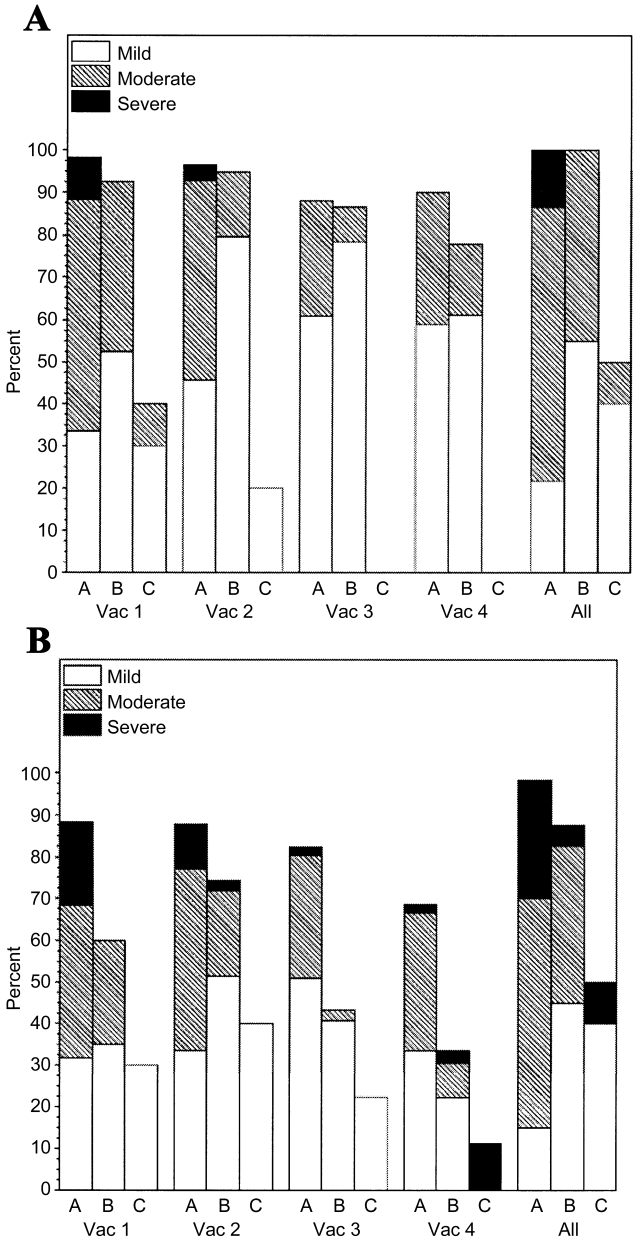

Figure 1. Comparison of local *(panel A)* and systemic *(panel B)* symptoms, between the high-dose vaccine–preparation group, the regular-dose vaccine–preparation group, and the placebo-preparation group. Data are for each of the 4 vaccinations (Vac 1–Vac 4) and for all of them combined.

tenderness, malaise, and myalgia. Although several observed side effects were graded as severe, all of these were transient and had either improved or resolved within 48 h after onset. With regard to abnormalities in laboratory results, there were no significant differences between the groups.

At the end of the study (1 year after the final vaccination), 45 (50.6%) of the 89 vaccine recipients were positive by ELISA (Abbott Laboratories); however, 44 (97.8%) of these 45 had indeterminate Western-blot results that were not considered positive by then-current diagnostic standards, and the 1 instance of reactive results on both ELISA and Western blot represented an HIV-infection event that occurred during the trial. High-dose recipients were significantly more likely to have a reactive result on ELISA (62%, compared with 33% of the regular-dose recipients) ($P = .01$).

In this trial, AEs judged to be probably or definitely due to the vaccine occurred in 11 participants; in 10 of these 11 cases, the AEs were due to severe constitutional symptoms consisting of chills, malaise, and fever (103.4°F in 2 participants), whereas the AE in the 1 other case was severe local-site injection pain. Remarkably, all of these AEs occurred in the recipients of the high-dose preparation, beginning <12 h after vaccination and either resolving or significantly improving by 48 h after vaccination.

Immunogenicity

CTL assay. A CTL assay performed with freshly isolated PBMCs 2 weeks after the last vaccination demonstrated that only a minor fraction in either group had detectable responses (table 3). No significant differences were noted between the high- and regular-dose groups. Of the 3 responders in the high-dose group, 2 responded to Gag only, and 1 responded to both Gag and Env; of the 5 responders in the regular-dose group, 2 individuals responded to Gag only, 2 responded to Nef only, and 1 responded to Env only.

IFN-γ ELISpot assay. As measured by IFN-γ ELISpot assay, the rates of response to any HIV protein were 9.6% (95% CI, 3.2%–21.0%), 5.4% (95% CI, 0.7%–18.2%), and 0.0% (95% CI, 0.0%–30.9%) for the high-dose, regular-dose, and placebo groups, respectively, at day 98 (i.e., 2 weeks after the third vaccination) (figure 2*A*). Two weeks after the last vaccination, the response rates had increased for the regular-dose group (to 14.7% [95% CI, 5.0%–31.1%]) but not for the high-dose group (8% [95% CI, 2.2%–19.2%]). By day 273, all responses had decreased, to 1.9% (95% CI, 0.1%–10.1%) for the high-dose group and to 0.0% (95% CI, 0.0%–11.6%) for the regular-dose group. The majority (67%) of the responses were to Gag, with the remainder being specific to Env and Pol (data not shown). Also, the magnitude of the responses was low and not signif-

Table 3. Point-prevalence CD8 CTL response rates at day 182.

Group	Response rate (% [exact 95% CI])
High dose	3/40 (8 [2–20])
Regular dose	5/31 (16 [5–34])
Placebo	0/7 (0 [0–41])

NOTE. CI, confidence interval; CTL, cytotoxic T lymphocyte.

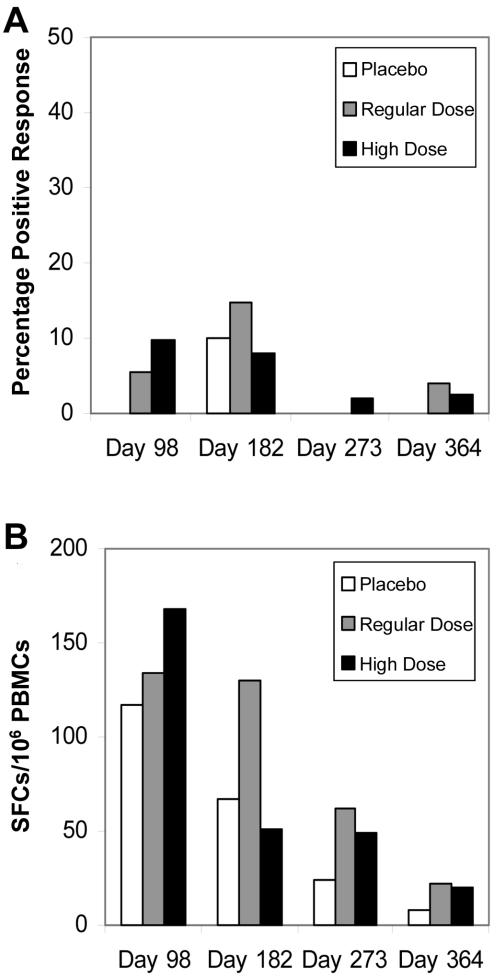

Figure 2. HIV-specific cytotoxic T lymphocyte response rates *(A)* and concentration of spot-forming cells (SFCs) *(B)*, as determined by interferon-γ ELISpot assay. Responses are to peptides representing Gag, Env, Nef, and the first half of Pol, at selected time points corresponding to 14 days after the third vaccination (Day 98) and 14 days (Day 182), 105 days (Day 273), and 196 days (Day 364) after the fourth vaccination. PBMCs, peripheral-blood mononuclear cells.

icantly greater than that of the responses seen in the placebo group (figure 2*B*).

HIV-specific antibodies. Anti-Gag antibody responses elicited by high- and regular-dose vCP1452 were measured by use of the qualitative screening and quantitative end-point ELISAs. By day 182, a total of 46% of the high-dose recipients had anti-Gag antibody responses, compared with 14% of the regular-dose recipients ($P = .002$) (table 4); by day 364 (6 months after the fourth vaccination), the antibody responses had dropped significantly. The mean binding-antibody titer was not significantly different between the 2 dose groups.

In both the regular- and high-dose groups, most recipients tested positive for neutralizing antibodies (in each case, the rate of response to HIV-1_{MN} was 92%), whereas all of the placebo recipients tested negative (table 5); however, the geometric mean titer (GMT) of neutralizing antibody was low (table 5). The 2 dose groups were not statistically significantly different in terms of $\log_{10}$ neutralizing-antibody titer ($P = .17$). Serum samples from 13 vaccine recipients and 5 placebo recipients were screened, at a 1:10 dilution, for neutralizing activity against 11 heterologous clade B HIV-1 strains from early seroconverters (Appendix B). A low (<16%) frequency of weak positive neutralization (i.e., 51%–76% neutralization) was detected in both of the dose groups.

Anti-canarypox antibodies. In view of the low frequency and magnitude of HIV-specific responses, we wanted to determine the immune responses directed against the vector. Anti-canarypox antibodies were measured both at baseline and 2 weeks after the last vaccination (figure 3). Although there was a trend toward increased anti-canarypox–binding antibodies at day 182, the difference was not statistically significant ($P = .08$).

DISCUSSION

Although recombinant HIV canarypox vaccines had been given to humans in numerous trials [3, 18, 19, 23, 28, 29], the maximum tolerated dose had not yet been determined; nor had it been determined whether immunogenicity could be achieved with higher doses of vaccine. The present trial has demonstrated that a 6-fold increase in vaccine dose ($10^{8.0}$ $TCID_{50}$) is poorly tolerated, resulting in an unacceptable level of local and systemic side effects, compared with either the regular dose ($10^{7.26}$ $TCID_{50}$) or placebo. Furthermore, the increased dose of vaccine did not translate into higher CTL response rates, as measured by 2 distinct assays.

The degree of toxicity that we achieved with this $\frac{3}{4}$-log increase in dose was surprising, because lower doses were well tolerated. In some instances, systemic symptoms necessitated

Table 4. p24-specific binding-antibody response rates, by ELISA.

Group	Response rate (% [exact 95% CI])	GMT
98		
High dose	9/44 (20 [10–35])	62.7
Regular dose	2/32 (6 [1–21])	51.0
Placebo	0/8 (0 [0–37])	…
182		
High dose	23/50 (46 [32–61])	59.4
Regular dose	5/37 (14 [5–29])	43.8
Placebo	0/10 (0 [0–31])	…
364		
High dose	3/44 (7 [1–19])	<50.0
Regular dose	1/25 (4 [0–20])	<50.0
Placebo	0/8 (0 [0–37])	…

NOTE. CI, confidence interval; GMT, geometric mean titer.

Table 5. Neutralizing-antibody response rates of HIV-1_{MN} at day 182.

Group	Response rate (% [95% CI])	GMT
High dose	47/51 (92 [81–98])	63.2
Regular dose	35/38 (92 [79–98])	47.5
Placebo	0/10 (0 [0–31])	<10.0

NOTE. CI, confidence interval; GMT, geometric mean titer.

bed rest and prevented normal activities. Fortunately, all symptoms either resolved or significantly improved ⩽48 h after onset. The fact that these AEs occurred ⩽12 h after vaccination, even after a single dose, implicated innate immune responses as playing a causative role. Indeed, toxicities lessened in frequency with subsequent doses, suggesting that the adaptive immune response to the vector actually alleviated some of the side effects associated with further injections.

Recombinant HIV canarypox vector had never been given at such a high dose or in such a large volume. Whether this contributed to the poor tolerability of the high-dose preparation remains unclear. However, the observed local and systemic reactogenicity profile of the regular-dose group, despite the high volume of vaccine administered, was similar to that observed in other clinical trials [3, 18, 19, 24]. We believe that the difference in formulation (to achieve a similar osmolarity, the high-dose preparation was formulated with SWFI, and the regular-dose preparation was resuspended in 0.9% NaCl) is an unlikely explanation for the observed differences in tolerability, especially because the control, or placebo, preparation, also prepared with either NaCl or SWFI, did not show an increased rate of reactogenicity.

Increasing the dose of the HIV recombinant vector, vCP1452, by 6-fold did not increase HIV-specific CTL responses as measured by either chromium-release assays of freshly obtained PBMCs or ELISpot assays of frozen PBMCs. In fact, in neither dose group were the responses statistically significantly different from those produced by placebo. Other trials using canarypox HIV recombinant vectors have demonstrated that HIV-specific T helper responses (as measured by HIV-specific lymphoproliferation assays) are present in the majority of vaccinated individuals [23, 30]; whether there are quantitative differences between HIV-specific $CD4^+$ T cells elicited by the 2 doses used in the present trial remains unclear, because the IFN-γ ELISpot assay is not optimal for the detection of T helper responses in vaccinated participants (authors' unpublished results).

The majority of participants in the present study had neutralizing antibodies against the T cell line–adapted virus, HIV_{MN}, from which the *env* portion of vCP1452 was derived, but these responses were low titer, rarely neutralized primary isolates (data not shown), and were not increased by higher doses of the vaccine. The frequency of Gag-specific binding antibodies was the only measured immune response higher in the high-dose group than in the regular-dose group, indicating some potential for increased immunogenicity, but the antibody titers induced in the present trial were low compared with those in HIV-infected individuals, which average 10^3–10^4 GMT (data not shown).

The underlying reasons for the poor CTL responses, by both the high- and the regular-dose preparations of vCP1452, are not clear. These responses appear to be lower than those observed in other trials, which have used a similar product (i.e., vCP205); however, the number of enrollees in all such trials has been small, and the differences between these trials are not likely to be statistically significant. It is possible that the prevention of apoptosis afforded by the E3L and K3L genes of vCP1452 resulted in the absence of cross-presentation of antigen, which may be an important mechanism of CTL priming [31]; however, these types of preclinical studies have not been performed with vCP1452. Furthermore, AVEG 034 compared vCP205 (which does not contain either E3L or K3L) with vCP1452 and did not observe differences between their CTL response rates (authors' unpublished data). Although we did not exclude individuals who had undergone earlier vaccinia immunization, data from another study using HIV recombinant canarypox vectors did not show a diminution of CTL response rates, compared with those in vaccinia-naive subjects [23]. The lack of increase in immunogenicity in the high-dose group, despite increasing systemic and local toxicity, was also disappointing. It is possible that adaptive immune responses directed against the vector backbone limited any increase in HIV-specific CTL responses that was afforded by the high-dose formulation. The possibility remains that anti-vector responses continued to eliminate the vector before gaining entry into the cell, thereby preventing a further increase in

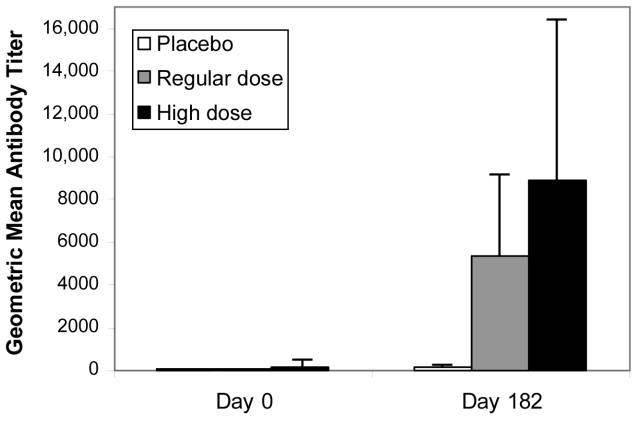

Figure 3. Anti-canarypox vector antibodies. IgG antibodies binding the CPpp antigen of canarypox were measured at baseline (Day 0) and after the fourth immunization (Day 182), by use of a standard ELISA. Error bars represent SD from the mean.

CTL responses; however, despite a trend toward higher anti-canarypox antibodies in the high-dose preparation than in the regular-dose preparation, no difference in the CTL response rate was observed.

We are not aware of any recombinant viral vector strategy that has demonstrated an increase in insert-specific CTL responses that is associated with an increased dose of the vaccine in humans—something that will be important to establish if recombinant-vectored vaccines are to be used in the future. Additional basic studies to improve the cellular immune response engendered by the canarypox vectors are needed. In addition, formulation and process improvements are necessary to design preparations that, if used at higher-dose levels, can provide a more acceptable safety profile.

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APPENDIX A

SUMMARY OF LOCAL AND SYSTEMIC SYMPTOMS OCCURRING AFTER AN INJECTION.

Symptom	High dose (n = 60)	Regular dose (n = 40)	Placebo (n = 10)
Pain			
None	3 (5)	3 (8)	8 (80)
Mild	13 (22)	21 (53)	1 (10)
Moderate	36 (60)	16 (40)	1 (10)
Severe	8 (13)	0 (0)	0 (0)
Tenderness			
None	2 (3)	0 (0)	6 (60)
Mild	19 (32)	24 (60)	4 (40)
Moderate	33 (55)	16 (40)	0 (0)
Severe	6 (10)	0 (0)	0 (0)
Pain and/or tenderness			
None	0 (0)	0 (0)	5 (50)
Mild	13 (22)	22 (55)	4 (40)
Moderate	39 (65)	18 (45)	1 (10)
Severe	8 (13)	0 (0)	0 (0)
Erythema			
None	48 (80)	30 (75)	10 (100)
>0–10 cm^2	5 (8)	6 (15)	0 (0)
>10–25 cm^2	0 (0)	2 (5)	0 (0)
>25 cm^2	7 (12)	2 (5)	0 (0)
Induration			
None	43 (72)	31 (78)	10 (100)
>0–10 cm^2	8 (13)	7 (18)	0 (0)
>10–25 cm^2	7 (12)	1 (3)	0 (0)
>25 cm^2	2 (3)	1 (3)	0 (0)

(continued)

Appendix A. ***(Continued.)***

Symptom	High dose (n = 60)	Regular dose (n = 40)	Placebo (n = 10)
Maximum erythema and/or induration			
None	41 (68)	26 (65)	10 (100)
>0–10 cm^2	8 (13)	9 (23)	0 (0)
>10–25 cm^2	3 (5)	2 (5)	0 (0)
>25 cm^2	8 (13)	3 (8)	0 (0)
Width of lymph nodes			
None	54 (90)	40 (100)	10 (100)
>0–1.4 cm	4 (7)	0 (0)	0 (0)
>1.4–3 cm	2 (3)	0 (0)	0 (0)
>3 cm	0 (0)	0 (0)	0 (0)
Malaise			
None	6 (10)	14 (35)	8 (80)
Mild	14 (23)	16 (40)	2 (20)
Moderate	28 (47)	9 (23)	0 (0)
Severe	12 (20)	1 (3)	0 (0)
Myalgia			
None	4 (7)	10 (25)	10 (100)
Mild	11 (18)	19 (48)	0 (0)
Moderate	36 (60)	10 (25)	0 (0)
Severe	9 (15)	1 (3)	0 (0)
Headache			
None	10 (17)	25 (63)	5 (50)
Mild	19 (32)	7 (18)	4 (40)
Moderate	27 (45)	6 (15)	0 (0)
Severe	4 (7)	2 (5)	1 (10)
Subjective fever			
None	7 (12)	25 (63)	9 (90)
Mild	15 (25)	10 (25)	1 (10)
Moderate	29 (48)	4 (10)	0 (0)
Severe	9 (15)	1 (3)	0 (0)
Nausea			
None	26 (43)	31 (78)	9 (90)
Mild	19 (32)	7 (18)	1 (10)
Moderate	14 (23)	2 (5)	0 (0)
Severe	1 (2)	0 (0)	0 (0)
Maximum systemic symptoms			
None	1 (2)	5 (13)	5 (50)
Mild	9 (15)	18 (45)	4 (40)
Moderate	33 (55)	15 (38)	0 (0)
Severe	17 (28)	2 (5)	1 (10)
Maximum temperature			
<100°F	13 (22)	30 (75)	10 (100)
100°F–100.9°F	17 (28)	8 (20)	0 (0)
101°F–101.9°F	21 (35)	1 (3)	0 (0)
>102°F	9 (15)	1 (3)	0 (0)

APPENDIX B

NEUTRALIZATION OF HETEROLOGOUS PRIMARY HIV-1 ISOLATES.

Patient	ID_{50} for MN[a]	Reduction in relative light units,[b] %										
		6101.10	QH0692.42	5768.4	3988.25	6535.3	BG1168.1	PVO.4	TRO.11	RHPA4259.7	REJO4541.67	THRO4156.18
059	283	13	18	29	0	25	7	30	14	10	42	13
086	326	38	**57**	22	0	27	5	**72**	19	15	32	0
054	265	0	6	NT	**76**	28	44	0	**57**	**57**	29	48
102	220	0	0	NT	0	NT	25	0	16	21	NT	19
088	231	24	37	NT	**53**	NT	39	**51**	**53**	**53**	49	36
050	446	0	9	7	12	19	10	13	26	26	27	19
092	489	0	19	9	28	0	0	4	22	3	22	0
009	276	0	21	0	0	17	0	0	0	0	28	0
062	366	4	14	31	18	44	0	26	18	23	14	0
089	253	0	35	26	3	14	0	26	12	11	32	12
108	275	0	11	7	4	21	4	0	18	7	28	12
085	41	21	36	19	23	4	13	31	0	48	36	48
017	16	0	0	0	0	0	0	0	18	20	25	4
048	<10	7	9	26	0	17	7	9	0	21	17	30
074	<10	0	19	14	4	14	16	12	21	26	18	25
082	<10	21	16	0	0	9	19	17	0	40	19	35
049	<10	0	0	35	0	0	10	0	0	10	9	9
044	<10	0	0	0	0	0	0	0	15	16	21	40

NOTE. NT, not tested.

[a] In assay of MT-2 cells. Values are the reciprocal of the serum dilution at which 50% of MT-2 cells are protected from virus-induced killing as measured by uptake of vital dye (neutral red).

[b] In assay of TZM-bl cells. Samples were screened at 1:10 dilution, in triplicate, against the virus strain indicated; all virus strains are molecularly cloned Env pseudotypes. Values are percent reduction relative to 1:10 dilution of corresponding preimmune serum at day 0.

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