Induction of Human Immunodeficiency Virus Type 1 (HIV-1)–Specific Cytolytic T Lymphocyte Responses in Seronegative Adults by a Nonreplicating, Host-Range–Restricted Canarypox Vector (ALVAC) Carrying the HIV-1_{MN} *env* Gene

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$CD8^+$ cytolytic T lymphocytes (CTL) are likely to be an important component of effective vaccines against human immunodeficiency virus type 1 (HIV-1). CTL can be induced most effectively with live virus vectors. However, because of concerns about the safety of such vectors, a nonreplicating canarypox vector (ALVAC) capable of expressing foreign genes in mammalian cells has been developed. This study evaluated the capacity of an ALVAC vector expressing the HIV-1_{MN} envelope (env) glycoprotein to induce HIV-1–specific CTL in seronegative volunteers. Protocols were designed to determine whether immunization with ALVAC alone or in combination with subunit boosting could induce CTL in vaccinia-immune and -naive volunteers. A simple method for antigen-specific in vitro stimulation was used to detect CTL responses in HIV-1–seronegative vaccine recipients. The results indicate that low doses of a nonreplicating virus vector alone can elicit both $CD4^+$ and $CD8^+$ HIV-1–specific CTL in a subset of seronegative volunteers.

The development of an effective vaccine against human immunodeficiency virus type 1 (HIV-1) may require immunization regimens that elicit both cytolytic T lymphocyte (CTL) and neutralizing antibody responses [1]. Attempts to induce HIV-1–specific $CD8^+$ CTL in humans by vaccination have had limited success. Purified recombinant HIV-1 env protein vaccines elicit $CD4^+$ CTL responses in a subset of persons who are immunized [2]. Because live virus vectors should in principle enable presentation of target antigens in association with class I major histocompatibility complex (MHC) molecules and induction of a $CD8^+$ CTL response, vaccine regimens involving live vaccinia virus vectors have recently been evaluated. HIV-1–specific $CD8^+$ CTL were detected in a fraction of vaccinia-naive volunteers tested but not in vaccinia-immune volunteers [3, 4]. However, safety concerns over the use of live vaccinia virus remain.

More recently, attention has focused on nonreplicating virus vectors, particularly members of the *Avipoxvirus* genus, such as the fowlpox and canarypox viruses. The canarypox virus is naturally host-range restricted and does not appear to replicate on mammalian cell substrates [5, 6]. Nevertheless, canarypox virus vectors (ALVAC) engineered to express extrinsic antigens can elicit protective immune responses to viral pathogens in nonavian species [5–7]. An ALVAC vector expressing the HIV-1 env glycoprotein induces $CD8^+$ CTL in mice [8], and an ALVAC vector carrying the rabies virus glycoprotein G gene is safe and immunogenic in humans [9]. The purpose of this study was to determine whether HIV-1–specific CTL responses could be induced in seronegative human volunteers by a vaccine regimen involving initial priming with a live recombinant canarypox virus expressing the HIV-1_{MN} *env* gene.

Materials and Methods

Vaccines. The vaccines tested included a live recombinant canarypox virus carrying the HIV-1_{MN} *env* gene (ALVAC-HIV; vCP125) and a placebo vaccine consisting of live recombinant canarypox virus carrying the rabies glycoprotein G gene (ALVAC-RG; vCP65). In these vectors, foreign gene expression is under transcriptional control of the vaccinia H6 early/late promoter. The ALVAC-HIV and ALVAC-RG vectors were produced by Pasteur Mérieux Serums et Vaccins (Marnes-la-Coquette, France). Some volunteers were boosted with purified recombinant HIV-1_{SF-2} gp120 in MF59 adjuvant (Chiron [Biocene], Emeryville, CA). The MF59 adjuvant is formulated as an emulsion containing polysorbate 89, sorbitan trioleate, and squalene.

Study design. HIV-1 env-specific CTL responses were measured in 17 HIV-1–seronegative adult volunteers participating

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Informed consent was obtained from all study participants. Human experimentation guidelines of the US Department of Health and Human Services and Johns Hopkins University were followed.

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in a phase I HIV-1 vaccine trial organized by the National Institute of Allergy and Infectious Diseases AIDS Vaccine Clinical Trials Network. Volunteers were divided into 2 groups on the basis of prior immunization with vaccinia virus. Twelve volunteers were primed at the beginning of the study (time 0) and 2 months later with 10^6 $TCID_{50}$ ALVAC-HIV and immunized at 9 and 12 months with either ALVAC-HIV (7 volunteers) or purified recombinant HIV-1 gp120 in MF59 adjuvant (5 volunteers). Five other volunteers were primed at time 0 and 2 months later with ALVAC-RG. These volunteers were immunized again at 9 and 12 months with ALVAC-RG (2 persons) or recombinant gp120 in MF59 (3 persons). Heparinized blood was collected immediately before and 2 weeks after the time 0 and 2- and 9-month immunizations. It was also collected 2 weeks after the 12-month immunization. CTL responses were analyzed in a blinded fashion. Preliminary results of safety and immunogenicity studies will be presented elsewhere.

Expansion of HIV-1–specific CTL. Peripheral blood mononuclear cells (PBMC) were plated in 24-well plates at 10^7/well in culture medium consisting of RPMI, 10% heat-inactivated fetal calf serum (Hyclone Laboratories, Logan, UT), 50 U/mL penicillin, 50 μg/mL streptomycin, and 4 m*M* L-glutamine. Cultures also included stimulator cells, which were prepared by infecting autologous Epstein-Barr virus (EBV)–transformed B lymphoblastoid cell lines (B-LCL) at an MOI of 5:1 with a WR strain vaccinia virus vector expressing the HIV-1_{MN} env glycoprotein (vac-env; vP1174). Cells were incubated overnight, washed, and resuspended at a concentration of 10^6 cells/mL in culture medium containing 10 μg/mL psoralen (4′-aminomethyl-4–5′-8-trimethylpsoralen hydrochloride; HRI Associates, Concord, CA). After a 15-min incubation in the presence of psoralen, the cells were transferred to a sterile petri dish and irradiated using a long-wave UV light box (transilluminator 4000; Stratagene, La Jolla, CA) for 5 min with occasional mixing. PBMC were plated at a concentration of 5×10^6 cells/mL with washed, psoralen-treated stimulator cells at a PBMC-to-stimulator ratio of 10:1. On day 4, interleukin (IL)-2 (Chiron) was added to a final concentration of 100 U/mL. Cultures were subsequently fed with culture medium containing 100 U/mL IL-2 as needed.

Detection of CTL responses. After a single 9-day in vitro stimulation, antigen-specific CTL activity was determined as previously described [3]. Target cells were mock-infected autologous B-LCL and autologous B-LCL infected with vac-env or a wild type control WR strain vaccinia virus (vac) as described above. Background lysis by vaccinia- and EBV-specific CTL was reduced to near zero in all cultures by the addition of a 30- to 40-fold excess of cold autologous vac-infected B-LCL. The SE of the percent specific lysis was calculated as previously described [10] and was generally <5%. The phenotype of the responding cells was determined by selective depletion of T cell subpopulations with anti-CD4 or -CD8 monoclonal antibodies and rabbit complement under conditions that gave >95% lysis of test populations of $CD4^+$ or $CD8^+$ lymphoblasts.

Results

A simple method for detecting vaccine-induced CTL. On the basis of previous experience detecting CTL in seropositive persons [11], we developed a simple method to detect CTL responses in HIV-1–seronegative vaccine recipients. To enhance detection of HIV-1–specific memory T cells, PBMC from vaccine recipients were stimulated in vitro with autologous vac-env–infected B-LCL. Preliminary studies established that vac-env–infected autologous B-LCL were superior to vac-env–infected autologous monocytes in inducing the proliferation of HIV-1–specific $CD8^+$ T cell clones (data not shown). Before coculture, the stimulating cells were treated with psoralen and UV light to inactivate vaccinia virus, thereby preventing vaccinia infection of the responding cells without causing denaturation of cell surface proteins involved in antigen presentation [12]. PBMC were cultured with stimulator cells for 9 days and then assayed for cytolytic activity.

In addition to stimulating env-specific CTL, this method may also activate vaccinia- and EBV-specific CTL, resulting in high background lysis. To circumvent this problem, excess unlabeled, vac-infected, autologous targets were included in all assays. The addition of up to a 50-fold excess of cold targets produced no detectable suppression of env-specific lysis by CD8+ CTL clones (data not shown); therefore, CTL assays were done in the presence of a 30- to 40-fold excess of vac-infected cold targets, a level that was sufficient to reduce the vaccinia- and EBV-specific background lysis to nearly zero.

env-specific CTL responses in vaccine recipients. All volunteers were negative for HIV-1–specific CTL before vaccination (table 1). Negative baseline CTL assays on cultures from a representative vaccinia-immune volunteer are shown in figure 1 (A, B). Cultures from vaccinia-immune volunteers showed significant background lysis in the absence of cold targets (figure 1A). The background lysis was attributed to vaccinia- or EBV-specific CTL (or both) and was dramatically reduced by the addition of excess cold targets (figure 1B).

env-specific CTL were detected 2 weeks after the second immunization in 2 of 12 volunteers primed with ALVAC-HIV and in none of 5 primed with the control ALVAC-RG vector. Assays were considered positive for env-specific CTL if the lysis of env-expressing targets was ≥10% higher than the lysis of control vac-infected targets at effector-to-target cell (E:T) ratios between 12:1 and 50:1. A representative positive CTL assay in the presence of cold targets is shown in figure 1C. To confirm these findings, repeat peripheral blood samples were drawn 4 weeks after the second immunization from both volunteers showing a positive CTL response, and CTL activity was measured again. Both volunteers showed env-specific CTL activity 4 weeks after the second immunization, indicating that the assay was highly reproducible and that response persisted for at least 4 weeks (figure 1D).

Phenotype and MHC restriction of effector cells. The phenotype of the env-specific effector cells was analyzed in parallel CTL assays by selective depletion of $CD4^+$ or $CD8^+$ cells

Table 1. Longitudinal analysis of env-specific CTL responses in volunteers first primed with ALVAC-HIV or ALVAC-RG and later immunized with ALVAC-HIV, gp120, or ALVAC-RG.

Subjects	Immunization		% env-specific lysis*			
	0/2 month	9/12 month	After 2nd vaccination	Before 3rd vaccination	After 3rd vaccination	Before 4th vaccination
Vaccinia immune						
85R	ALVAC-HIV	ALVAC-HIV†	5.3	ND	4.3	NG‡
86B	ALVAC-HIV	ALVAC-HIV	9.5	−3.4	−5.7	−1.0
86H	ALVAC-HIV	ALVAC-HIV	−2.2	−9.7	9.9	0.2
86L	ALVAC-HIV	ALVAC-HIV†	3.6	ND	28.6	10.7
87I	ALVAC-HIV	ALVAC-HIV	NG	5.3	0.5	NG
87C	ALVAC-HIV	gp120	−4.3	−0.2	−0.4	1.9
88J	ALVAC-RG	gp120	2.1	−7.7	0.4	HB§
8A9	ALVAC-RG	gp120	−3.7	−13.6	−5.4	1.2
85Y	ALVAC-RG	gp120	−1.2	3.6	−1.7	1.8
86R	ALVAC-RG	ALVAC-RG	0.5	NG	5.4	3.6
Vaccinia naive						
86Z	ALVAC-HIV	ALVAC-HIV	0.5	−4.1	0.3	NG
86P	ALVAC-HIV	ALVAC-HIV	3.1	−18.1	1.3	−4.3
85X	ALVAC-HIV	gp120	5.2	5.0	−1.7	3.6
86F	ALVAC-HIV	gp120	33.9	ND	4.2	HB
88X	ALVAC-HIV	gp120	49.5	5.8	15.6	7.7
8BE	ALVAC-HIV	gp120	HB	−6.1	5.0	0.1
886	ALVAC-RG	ALVAC-RG	−5.2	2.8	−2.0	0.6

NOTE. ALVAC-HIV, live recombinant canarypox virus carrying HIV 1_{MN} *env* gene; ALVAC-RG, placebo consisting of live recombinant canarypox virus carrying rabies glycoprotein G gene; gp120, subunit vaccine consisting of HIV-1_{SF-2} gp120 (50 μg) in MF59.

* Determined by subtracting % specific lysis of control vaccinia (vac)-infected targets from % specific lysis of vac-env–infected targets. CTL assays were done 2 weeks before or after vaccination, as indicated. Responses considered positive (% lysis >10) are underlined. ND, not done.

† Third vaccine given at 6, not 9, months.

‡ NG, no growth; assay not done due to insufficient expansion of effector cells.

§ Assay was inconclusive due to high background (HB) lysis.

by antibody-dependent, complement-mediated lysis prior to mixing with target cells. With the depletion of $CD4^+$ T cells, there was a small but detectable decrease in env-specific CTL activity at the lowest E:T ratios, indicating that $CD4^+$ CTL contribute to the observed lytic activity seen in bulk culture (figure 1E). With depletion of $CD8^+$ T cells, there was a larger decrease in env-specific lysis, which was apparent even at high E:T ratios, indicating that $CD8^+$ env-specific CTL are responsible for most of the observed env-specific lysis (figure 1F). When cultures that were positive for env-specific CTL activity on autologous targets were assayed on MHC-mismatched targets expressing the HIV-1 *env* gene, env-specific lysis was not detected, indicating that the env-specific effectors were fully MHC restricted (figure 1G). These data indicate that the env-specific cytolysis was mediated by both $CD4^+CD8^-$ MHC class II–restricted and $CD4^-CD8^+$ MHC class I–restricted CTL.

Longitudinal analysis of CTL responses. Table 1 presents a summary of the env-specific CTL activity in stimulated cultures from all volunteers. In cultures positive for CTL activity, significant cell growth was consistently noted. In 5 cultures, no proliferation occurred, and after 9 days, insufficient cells remained for analysis of CTL activity. These cultures were presumably negative for env-specific CTL activity. After two primings with ALVAC-HIV, high levels of env-specific CTL activity were detected in 2 of 12 volunteers. Both subjects were vaccinia-naive. No HIV-1–specific CTL activity was detected in volunteers who received the control ALVAC-RG vector. Seven ALVAC-HIV–primed volunteers received two additional immunizations with the vaccine; 1 subject (86L) who was vaccinia immune and previously CTL negative had high levels of HIV-1–specific CTL activity after the third immunization and detectable activity after the fourth. The other 5 volunteers were boosted at 9 and 12 months with purified rgp120; 1 subject (88X) who had high levels of env-specific CTL activity after the second immunization with ALVAC-HIV, had detectable env-specific CTL activity after but not immediately before the first rgp-120 boost. No env-specific CTL activity was detected in the 3 volunteers previously primed with ALVAC-RG and boosted with rgp-120.

Discussion

In this study, vaccination of HIV-1–seronegative persons with a live recombinant canarypox virus expressing the HIV-

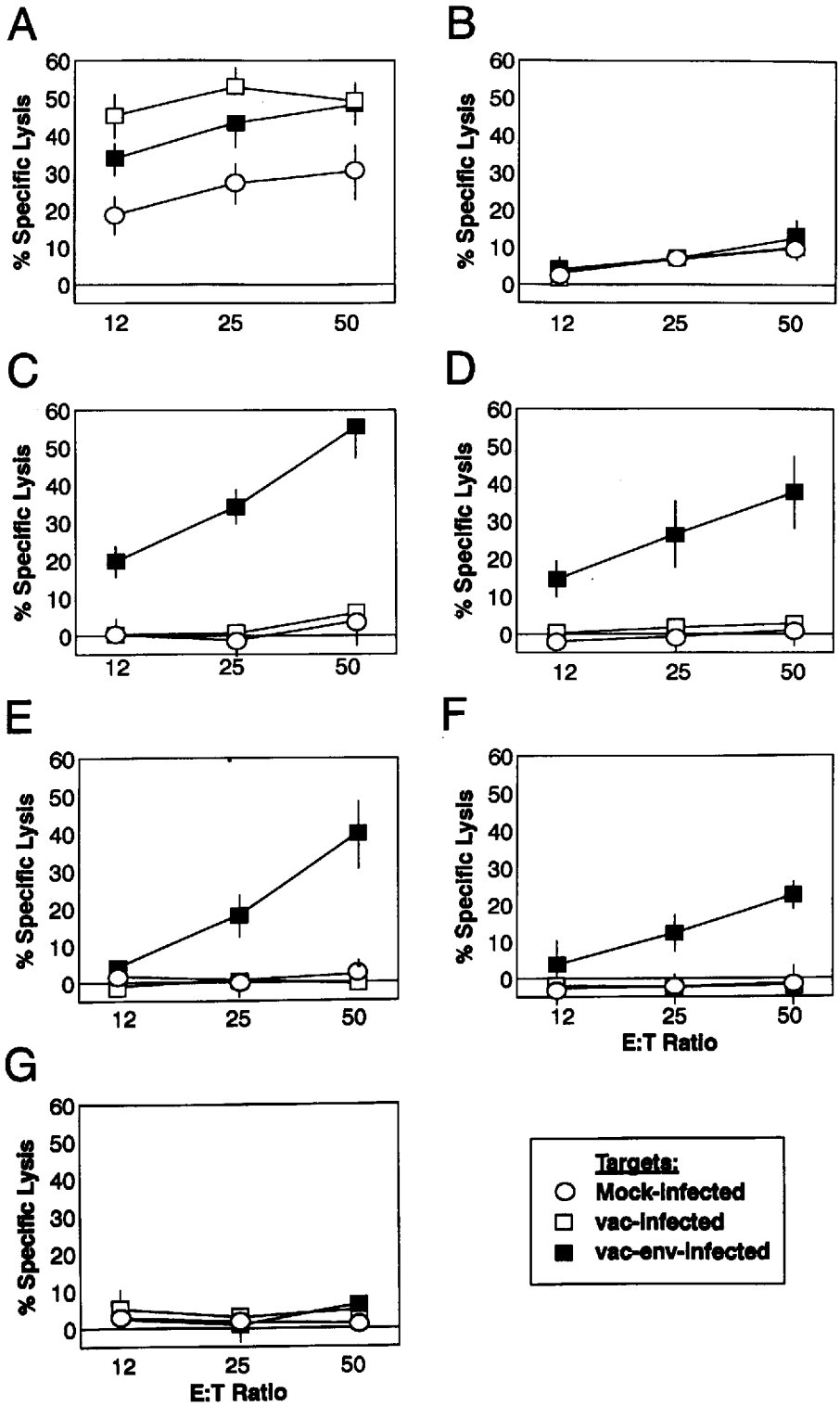

Figure 1. Induction of env-specific CTL activity by immunization with live recombinant canarypox virus carrying HIV-1_{MN} *env* gene (ALVAC-HIV). Peripheral blood mononuclear cells (PBMC) from volunteers were stimulated in vitro for 9 days with autologous B lymphoblastoid cell lines (B-LCL) that had been infected for 12 h with a WR strain vaccinia virus vector expressing the HIV-1_{MN} *env* glycoprotein (vac-env) and then treated with psoralen and UV light. CTL activity was measured in a standard 6-h ^{51}Cr release assay at indicated effector-to-target cell (E:T) ratios. Targets were autologous B-LCL that were mock infected, infected with vac, or infected with vac-env. Assays were done in absence (**A**) or presence (**B-G**) of 30- to 40-fold excess of vac-infected, cold B-LCL targets. Results are mean ± SE of % specific lysis, calculated as described [10]. **A**, Representative baseline CTL assay on stimulated PBMC from vaccinia-immune volunteer in absence of cold targets. **B**, CTL assay on same culture in presence of cold targets. **C**, Representative positive CTL assay on stimulated cultures of PBMC isolated from volunteer 88X 2 weeks after second immunization with ALVAC-HIV. **D-G**, env-specific CTL activity in stimulated cultures of PBMC isolated from volunteer 88X 4 weeks after second immunization with ALVAC-HIV. **D**, Untreated effector cells. **E**, Residual CTL activity in effector cells pretreated with anti-CD4 and complement. **F**, Residual CTL activity in effector cells pretreated with anti-CD8 and complement. **G**, CTL activity of untreated effector cells against major histocompatibility complex-mismatched targets.

1 *env* gene induced HIV-1 env-specific $CD4^+$ and $CD8^+$ CTL. Similar results have been reported in human vaccine trials involving replication-competent vaccinia vectors [3, 4]. The unique aspect of the present study is that the vaccine vector was not capable of replicating in the host. In this situation, the number of host cells capable of presenting the vaccine antigen with class I may be limited to the number of cells that initially become nonproductively infected with the

vaccine vector. It is therefore encouraging that systemic CTL responses can be detected in some volunteers after immunization with only 10^6 $TCID_{50}$ ALVAC-HIV. In each of the volunteers with a positive CTL response, the response could be attributed to immunization with ALVAC-HIV. Baseline CTL assays were uniformly negative. Two vaccinia-naive volunteers had CTL responses after the two immunizations with ALVAC-HIV. Of significance, repeated immunization with ALVAC-HIV also induced env-specific CTL in 1 of 5 vaccinia-immune volunteers, indicating that prior exposure to a related poxvirus does not preclude response to canarypox virus–based vaccines.

These results provide direct support for the concept that nonreplicating virus vectors can induce CTL in humans. These results are of particular significance with respect to the development of an AIDS vaccine in light of safety considerations inherent in the use of replication-competent virus vectors to induce CTL. The overall frequency of CTL responses to ALVAC-HIV is not dissimilar to the frequency of CTL responses to other candidate AIDS vaccines [2–4]. Additional studies will be required to determine whether alterations in the dose or immunization schedule can increase the frequency of positive responses. The inclusion of additional HIV-1 genes in the vaccine vector may also increase the proportion of responders. The method for in vitro stimulation and detection of vaccine-induced CTL in HIV-1–seronegative volunteers described here may be useful in the evaluation of other vaccines.

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