A Vaccinia-gp160-Based Vaccine But Not a gp160 Protein Vaccine Elicits Anti-gp160 Cytotoxic T Lymphocytes in Some HIV-1 Seronegative Vaccinees

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Summary: Cytotoxic T lymphocytes (CTL) play an important role in the immune response to viral infection by recognizing and destroying infected cells. HIV-1 elicits an unusually strong CTL response in infected individuals and clearance of the viremia of acute infection coincides with the development of HIV-specific CTL. Because HIV-specific CTL may provide protection against de novo viral infection, we compared the CTL response in seronegative volunteers treated with two vaccination approaches. Seven volunteers were immunized with a live recombinant vaccinia virus expressing the HIV envelope protein $gp160_{LAI}$ (HIVAC-1e) and boosted with 640 μg recombinant baculovirus-expressed $gp160_{LAI}$ in alum 1–13 months later. In a second study, three volunteers underwent four successive immunizations with 640 μg subunit $gp160_{LAI}$ in alum at 0, 1, 6, and 12 months. The first vaccination strategy using a live vector would be expected to generate gp160-specific CTL, while for the second, using only whole-protein subunit, the generation of specific CTL would be unlikely. Predominantly $CD8^+$ T-cell lines generated from PBMC by nonspecific stimulation with PHA and IL-2 were screened after three to four weeks of culture for cytolytic activity against autologous targets infected with vaccinia vectors encoding env_{LAI}, *RT, gag,* and *lacZ* control. A strong gp160-specific CTL response was detected in two vaccinees in the recombinant vaccinia plus subunit boost study. Modest responses were seen in four of the other five live vector-primed vaccinees. No significant gp160-specific CTL were observed in three volunteers given only subunit rgp160 or in five control subjects. **Key Words:** HIV—Vaccine—Cytotoxic T lymphocytes—gp160.

The development of an effective vaccine to protect against HIV-1 infection is a high priority. Generation of neutralizing antibodies has been the cornerstone of vaccine design for most infections, but disappointing results in animal models with HIV-1 vaccines designed primarily to optimize neutralizing antibody production suggest that neutralizing antibodies may not be sufficient to protect against HIV-1 infection. Because humoral immunity can be studied with frozen sera, whereas cellular immunity assays require live effector as well as autologous stimulator/target cells, many studies of vaccine-stimulated immunity have focused on humoral immunity.

Little is known about which arms of the immune system are necessary to provide protective immunity against de novo infection. Cell-mediated immunity is, however, known to be central to the immune response once a viral infection has become established. Cytotoxic T lymphocytes (CTL), which recognize and lyse infected cells, play an important role in viral clearance [1]. HIV-1 elicits an unusually strong CTL response in infected individuals and the appearance of HIV-specific CTL coincides with the resolution of the viremia associated with

acute infection (2). Progression from asymptomatic infection to AIDS may coincide with the loss of HIV-specific T-cell cytotoxicity (3–6). The importance of viral-specific CTL in containing established viral infections suggests that viral-specific $CD8^+$ memory T cells may also contribute to protection against de novo infection.

Vaccines vary in their ability to stimulate different aspects of immunity. T cells recognize antigenic peptides presented by major histocompatibility complex (MHC) molecules. The intracellular processing of antigen to generate the peptide–MHC complex differs for MHC class I (recognized by $CD8^+$ cells) and class II molecules (recognized by $CD4^+$ cells). The current paradigm for antigen presentation to class I- and class II-restricted T cells predicts that $CD8^+$ T cells will be stimulated primarily by antigens generated intracellularly and degraded in the cytosol, whereas $CD4^+$ T cells will be activated by antigens that bind to cell surface molecules and are endocytosed and degraded into peptides in lysosomal compartments (7). To elicit a T-lymphocyte response, a peptide must be properly processed, be able to bind strongly enough to the MHC to compete effectively with other peptides, and be recognized as a peptide–MHC complex by T cells in the repertoire (7–12). This model of dual pathways for antigen processing suggests that whole protein vaccines will be able to elicit $CD4^+$ (but not $CD8^+$) T-cell immunity as well as humoral immunity, whereas vaccines based on recombinant vectors that infect cells and express intracellular antigens will be able to stimulate both types of T cells but may be less effective at stimulating antibody production (13).

To test the validity of this model for human vaccination, we have analyzed the ability of two different vaccination strategies to elicit CTL against the HIV-1 envelope (env) glycoprotein gp160. HIV-1 seronegative vaccinia-naive volunteers were vaccinated either with live recombinant vaccinia-gp160 followed by a single boost with subunit gp160 (14) or with four immunizations of subunit gp160 (15). Only the first vaccination protocol would be expected to be effective at eliciting $CD8^+$ gp160-specific T cells.

MATERIALS AND METHODS

Vaccination

HIV-1 seronegative volunteers were recruited for participation in this study at the AIDS Vaccine Evaluation Unit in the Center for Immunization Research, School of Hygiene and Public

Health, Johns Hopkins University. The subjects were participants in two multicenter, randomized, double-blind phase 1 vaccine trials organized by the NIAID AIDS Vaccine Clinical Trials Network. Both studies were approved by the institutional Human Investigation Review Board and informed consent was obtained from each subject. In study 003B, participants received four intramuscular immunizations of 640 μg of baculovirus-produced subunit $gp160_{LAI}$ in alum at 0, 1, 6, and 12 months as published (15). Participants in study 002, who were required to be vaccinia-naive, were vaccinated by intradermal scarification with dilutions of a vaccinia-$gp160_{LAI}$ recombinant (HIVAC-1e; Oncogen/Bristol-Myers Squibb, Seattle, WA, U.S.A.), and boosted i.m. (1–13 months later) with 640 μg baculovirus-produced recombinant subunit $gp160_{LAI}$ in alum (VaxSyn; MicroGeneSys Inc., Meriden, CT, U.S.A.), as previously described (14,16). In three subjects (002A24, 002A28, and 002A29), no primary take was observed after the first immunization with the recombinant vaccinia, and they underwent a second vaccination with the vector. The preparation of the vaccinia vector expressing gp160 (17) and of the recombinant subunit gp160 expressed in baculovirus have been described elsewhere (15). Subject immunization schedules are shown in Fig. 1. Peripheral blood from five HIV-seronegative donors was also obtained with informed consent and approval of the New England Medical Center Human Investigation Review Committee.

Cell Lines

T-cell lines were generated by adding 2 μg/ml PHA-P (Difco Laboratories, Detroit, MI, U.S.A.) to peripheral blood mononuclear cells (PBMC) obtained by Ficoll-Hypaque density centrifugation from heparinized blood. In some instances density-separated PBMC were cryopreserved in fetal calf serum (JRH Biosciences, Lenexa, KS, U.S.A.) supplemented with 5% DMSO using a programmed cell freezer (Cryomed). Fresh or thawed cryopreserved cells were incubated at 5×10^5/ml in T-cell media, RPMI 1640 supplemented with 15% fetal calf serum, 200 U/ml rhuIL-2 (Cetus), 2 mM glutamine, 2 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μM β-mercaptoethanol. Twice a week the cultures were adjusted to 5×10^5/ml with fresh IL-2-containing media. Autologous B cell lines were generated simultaneously using B95-8 marmoset cell line supernatant by standard methods (18).
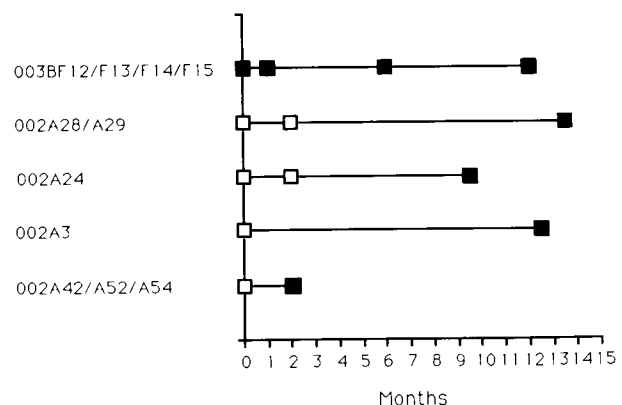

FIG. 1. Vaccination schedules for study 002 (HIVAC-1e and subunit rgp160) and study 003B (subunit rgp160). □, HIVAC-1e; ■, subunit gp160.

Immunomagnetic Depletion of T-Cell Lines

T cells to be CD4+ depleted were washed and suspended in 100 μl of PBS at 4°C and an excess of washed Dynabeads M-450 CD4 (Dynal, Great Neck, NY, U.S.A.) (10–20× the number of estimated CD4+ cells to be cleared) was added at 1.4×10^8 beads/ml in PBS–2% FCS. The magnetic bead–cell mixture was allowed to incubate with rocking for 30 min at 4°C, and the tube was placed in a magnetic particle concentrator for 3 min. The depletion was repeated on the nonadherent supernatant. An aliquot of the CD4+-depleted cell line was analyzed by flow cytometry to verify the extent of depletion (19). Analogous methods were used to deplete CD8+ cells with Dynabeads coupled to CD8 antibodies.

Flow Cytometry Analysis of T-Cell Lines

T-cell lines (2–10 × 10^5) of >90% viability were harvested and resuspended in 50 μl of cold FACS media (PBS with 1% BSA and 0.02% sodium azide) to which 5 μl of Leu2a-PE and 5 μl of Leu3a + 3b-FITC (Becton Dickinson, Mountain View, CA, U.S.A.) or other conjugated MAb were added. After incubation for 20 min at 4°C, the cells were washed with 1 ml of FACS media, pelleted, and washed again with 500 μl. Before two-color flow cytometry, the cells were fixed with 1% formaldehyde in FACS media. Previously phenotyped CD4+ and CD8+ clones were stained simultaneously to establish gates and gain settings.

Vaccinia Vectors

Vaccinia vectors encoding lacZ (vSC8), gp160 of the BH8 isolate of HIV-1$_{LAI}$ (vPE16), all but the last 22 residues of HIV-1 reverse transcriptase (RT) of subclone HXB.2 (vCF21), and the HXB.2 gag (HIV-1 capsid gene) (vDK1) were used to screen cell lines for specific cytotoxicity against gp160-, RT-, and gag-expressing targets (20–22). The HIV-1 sequences were inserted downstream from the 7.5 early–late promotor of vaccinia. The vaccinia virus preparations were titered by plaque assay on CV-1 cells (23).

Chromium Release Assay

Cytotoxicity assays were performed against target cells that were infected with vaccinia constructs encoding HIV-1 proteins or lacZ control. For infection, 2–5 pfu/cell of vaccinia virus was added to 5 × 10^5 exponentially growing B cells in 500 μl in a 24-well plate. The plate was incubated at 37°C over CO_2 with rocking for the first hour. After 16 h the cells were harvested and resuspended in 200 μl of serum-containing media to which 100–200 μCi of Na_2 ($^{51}CrO_4$) (Du Pont, NEN Research Products, Boston, MA, U.S.A.) was added. After incubation for 1 h at 37°C with occasional mixing, the targets were washed three times and resuspended at 10^5/ml. Labeled targets (10^4) were added to triplicate wells of U-bottom microtiter plates. Effector cells were suspended at various E:T ratios in 100 μl and added to target cells, and the plates were incubated at 37°C over CO_2 for 4 h. For each target, spontaneous release (SR) was determined from wells to which 100 μl media was added and total release (TR) calculated from wells containing 100 μl 1% Nonidet P-40. Supernatants (75 μl) from each well were counted on a gamma counter after addition of 100 μl 1% Nonidet P-40. Percent specific cytotoxicity was calculated from the average counts per minute (cpm) as [(average cpm −SR)/(TR − SR)] × 100. Spontaneous

release was below 20% of total release. The gp160-, gag-, or RT-specific cytotoxicity was defined as the difference between the percent specific cytotoxicity against gp160-, RT-, or gag-expressing targets and that against lacZ-expressing targets.

Statistical Methods

For each cell line tested, results from two or more experiments were pooled by calculating the average specific cytotoxicity and the pooled standard error. The average specific cytotoxicity and standard error were compared by Student's *t* test.

RESULTS

gp160-Specific Cytotoxicity in T-Cell Lines

After three to four weeks of culture, T-cell lines generated by nonspecific stimulation from PBMC were screened by ^{51}Cr-release assay for cytolytic activity against autologous B-lymphoblastoid cell lines (B-LCL) infected with vaccinia vectors encoding env$_{LAI}$, RT, gag, and lacZ control. The cytotoxicity assays for each effector T-cell line were repeated at least once and the amount of gp160-specific cytotoxicity was stable over the two-week period that the assays were performed. Because of the high concentrations of rhuIL-2 (200 Cetus U/ml) used to expand the T-cell lines, these cell lines were predominantly CD8+. The percent CD8+ cells in the lines as determined by flow cytometry was comparable whether the line was generated from normal unimmunized volunteers (77–85%), volunteers in the 003B recombinant gp160 protocol (54–78%), or volunteers in the 002 vaccinia-env plus boost protocol (59–73%). An unequivocal gp160-specific CTL response (>10% above background lysis of the vaccinia-lacZ infected target at an E:T ratio of 25:1) was detected in two HIVAC-1e plus subunit gp160 recipients (002A28 and 002A42) at 19 and five weeks after subunit boosting (Fig. 2, Table 1). Modest but statistically significant responses (5–10% above background at an E:T ratio of 25:1) were seen at least for one time point in four of the five other vaccinees in study 002. Since the cell lines were not specifically selected in vitro for gp160-specific cytotoxicity, the presence of even small degrees of cytotoxicity probably represents a significant frequency of circulating memory CTL responding to the antigen. No significant gp160-specific CTL were observed in three volunteers given only subunit gp160 or in five control subjects. T cells from a third subject (003BF12), who received adjuvant only as control in the subunit gp160 study, showed modest but significant gp160-specific cytotoxicity. The presence of gp160-specific CTL in occasional HIV-
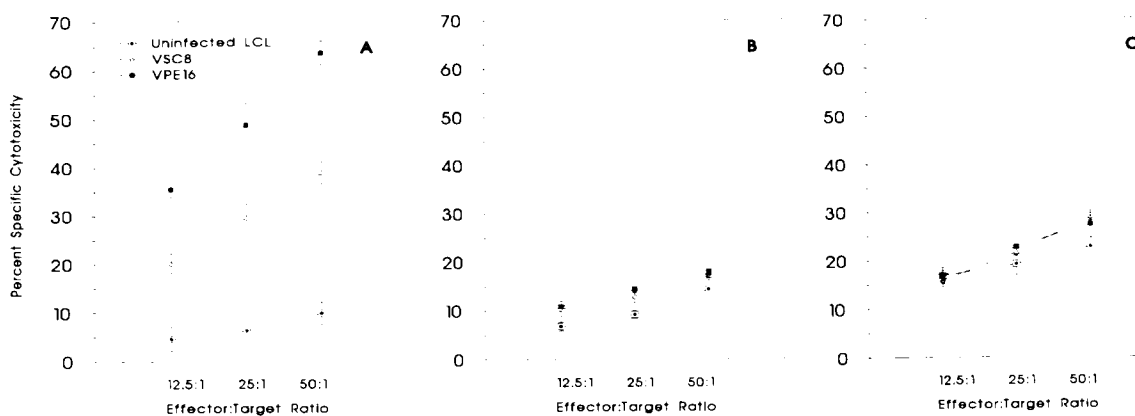
FIG. 2. HIV-1 envelope-specific CTL in representative vaccinees and an untreated control subject. PBMC from vaccinees and controls were stimulated with PHA, cultured for three to four weeks and assayed for cytotoxicity against autologous B-LCL that were either uninfected or expressed gp160 (vPE16) or lacZ (vSC8) control in a 4-h ^{51}Cr-release assay. Representative T-cell lines are shown from subjects **(A)** vaccinee 002A42, a HIVAC-1e and subunit rgp160 recipient, at five weeks after boosting, **(B)** subunit gp160 recipient 003BF15 studied two weeks after the fourth immunization, and **(C)** nonimmunized seronegative control NEMC002. The lysis of the uninfected target represents a possible combination of LAK-like activity, autoreactivity or recognition of the EBV-transformed phenotype. The difference between lysis of the vSC8-infected target and the uninfected B-LCL represents vaccinia-specific cytolysis, present to a significant degree only in the individual vaccinated with HIVAC-1e in **A**. The difference in lysis of vPE16- and vSC8-infected autologous targets represents gp160-specific cytolysis, also present only in **A**.

seronegative subjects has previously been reported (24). However, neither of the two HIVAC-1e recipients for whom prevaccination samples were available had CTL that recognized gp160-bearing targets prior to vaccination.

For some of the vaccinees, multiple blood samples were obtained and analyzed during the trial to evaluate the time course of the CTL response (Table 1). There was no clear enhancement of CTL response to gp160 after boosting with gp160 protein in the two vaccinees for which all time points were available (002A52 and 002A54). However, T-cell lines from these subjects had only modest gp160-specific CTL activity after the primary vaccination. In the two vaccinees with a high gp160-specific CTL response, the samples tested were collected after subunit boosting, and we are unable to fully define the effect on the CTL response of subunit boosting. The presence of detectable circulating gp160-specific CTL without specific in vitro selection as late as 19 weeks after boosting in one patient (002A28) is indeed surprising since the CTL response to influenza infection is generally undetectable after one month without in vitro stimulation to detect persistent circulating memory CTL (25).

In addition to the gp160-specific response, vaccination with HIVAC-1e induced a vaccinia-specific CTL response ranging from less than 10% in four vaccinees to above 20% in two recipients at an E:T ratio of 25:1 (Fig. 2A). One vaccinee had an intermediate vaccinia-specific response of 13%. In vac-

cinee 002A52, the vaccinia response was highest two weeks after receiving HIVAC-1e (23.5% at an E:T ratio of 25:1) and fell rapidly by week 4 after vaccination to less than 5%. In the other vaccinee for which all time points were available (002A54), the maximum vaccinia response was seen four weeks after vaccination (5.4% at an E:T ratio of 25:1).

Analysis of CD4$^+$ and CD8$^+$ gp160-Specific CTL Response

CD4$^+$ and CD8$^+$ gp160-specific CTL responses were analyzed separately in subject 002A28, whose T cells had significant env-specific cytotoxicity. Depletion of CD4- and CD8-expressing T-cell populations by immunomagnetic separation demonstrated specific cytolysis of env-expressing autologous B-LCL by both CD4$^+$ and CD8$^+$ cells of comparable extent (Fig. 3). The background lysis of the EBV-transformed LCL was mediated only by CD8$^+$ T cells, but the vaccinia-specific cytolysis in this patient was due to both CD4$^+$ and CD8$^+$ effector cells.

T-cell lines from the three vaccinees immunized with only subunit gp160 demonstrated no significant gp160-specific cytotoxicity. These cell lines were shown by FACS analysis to consist predominantly of CD8$^+$ cells (54–78%). Since subunit immunization would be expected to stimulate predominantly CD4$^+$ CTL, we assayed for gp160-specific cytotoxicity in a CD4-enriched subpopulation, isolated by immunomagnetic depletion of CD8$^+$ cells. No spe-

TABLE 1. *Gp160-specific cytotoxicity of nonspecifically stimulated T-cell lines generated from blood of vaccinees and control subjects obtained before or after vaccination*[a]

Vaccination protocol	Vaccinee	Time in relation to vaccination[b]	GP160-specific cytotoxicity (average mean difference ± pooled standard error[c])	p value for specific cytotoxicity
HIVAC-1e + subunit gp160 boost	002A3	V + 56W	4.8 ± 1.6	0.02
		B + 2W	6.5 ± 0.4	0.0002
		B + 4W	1.4 ± 3.4	0.70
	002A24	V + 2W	−5.7 ± 2.5	0.04
		V2 + 25W	4.9 ± 0.9	0.002
		B + 4W	3.0 ± 2.5	0.31
		B + 12W	8.1 ± 1.3	0.0036
	002A28	B + 19W	11.2 ± 0.9	1.2×10^{-7}
	002A29	V2 + 53W	3.5 ± 0.8	0.003
		B + 20W	2.5 ± 1.0	0.025
	002A42	B + 5W	19.1 ± 5.3	0.023
	002A52	I	0.0 ± 0.5	1.00
		V + 2W	5.3 ± 1.1	0.002
		V + 4W	3.0 ± 0.7	0.003
		B + 2W	3.7 ± 0.8	0.0007
		B + 4W	6.6 ± 0.8	1.2×10^{-7}
	002A54	I	0.5 ± 0.7	0.52
		V + 1W	7.3 ± 0.9	0.0016
		V + 4W	3.7 ± 1.1	0.01
		B + 2W	6.6 ± 1.1	0.0003
		B + 4W	6.0 ± 0.8	0.0017
Subunit gp160	003BF13	B3 + 4W	1.9 ± 0.9	0.06
		B3 + 26W	1.8 ± 1.1	0.13
		B4 + 2W	1.2 ± 0.8	0.16
	003BF14	B3 + 4W	2.2 ± 2.1	0.32
		B3 + 26W	−0.5 ± 1.1	0.64
		B4 + 2W	4.1 ± 1.8	0.045
	003BF15	B4 + 2W	0.7 ± 1.6	0.67
Control subjects				
Adjuvant only	003BF12	B4 + 2W	6.2 ± 0.7	8×10^{-6}
No vaccination	NEMC001	—	1.1 ± 0.7	0.13
	NEMC002	—	−0.6 ± 2.7	0.83
	NEMC003	—	−0.6 ± 1.1	0.6
	NEMC004	—	3.8 ± 1.4	0.02
	NEMC005	—	4.8 ± 1.7	0.02

[a] Results of cytotoxicity assays at an E:T ratio of 25:1 are given, although all assays were done at E:T ratios of 12.5, 25, and 50:1 with expected increases in cytotoxicity with increasing E:T ratios.
[b] I: initial; V: vaccination with HIVAC-1e; B: vaccination with subunit rgp160; W: week.
[c] The average mean difference between the HIV-1 envelope-specific and lacZ control lysis at an effector to target ratio of 25:1.

cific CD4^{+}-mediated cytolysis was detected in any of the three vaccinees immunized with subunit gp160 (Fig. 4).

DISCUSSION

The development of an effective vaccination strategy for HIV-1 infection has proven more challenging than initially expected. Few vaccines have provided protective immunity in the most relevant animal models—SIV infection in macaques or HIV-1 infection in chimpanzees. Early approaches focused primarily on eliciting a potent neutralizing antibody response. One of the few promising approaches, vaccination with a live HIV-expressing vaccinia vector followed by subunit boost, differs from others in its potential ability to stimulate viral-

specific CD4 and CD8 T cells as well as neutralizing antibodies. This approach has been shown to provide protection from challenge in the SIV macaque model. Initially, protection in macaques was demonstrated using SIV env-vaccinia immunization followed by env protein subunit boosting (26). More recently, protection has been demonstrated using immunization with live SIV-expressing vectors followed by boosting with pseudoviral particles that induced only low neutralizing antibody titers (S.-L. Hu, presented at Vaccine: Immune-Based Treatment Strategies for HIV, IBC meeting, Bethesda, MD, U.S.A., 1994). This result suggests that neutralizing antibody production may not be essential to the protection provided by these regimens. Because of a paucity of information available about the
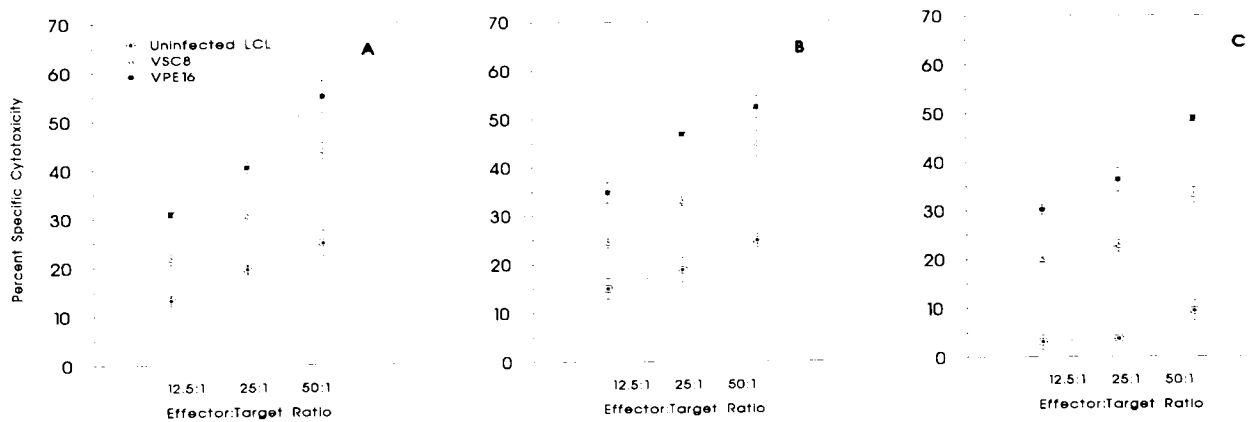
FIG. 3. Both CD4^{+} and CD8^{+} T cells from HIVAC-1e and subunit gp160 recipient 002A28 exhibit HIV-1 envelope-specific CTL. T-cell lines were generated from PBMC obtained 19 weeks after the subunit gp160 boost. On the day of the cytotoxicity assay, the unmanipulated T-cell line (A), CD4-depleted (B), or CD8-depleted (C) T-cell lines were tested for cytolysis of uninfected, gp160-expressing vaccinia-infected (vPE16), or vaccinia-lacZ-infected control (vSC8) autologous B-LCL targets.

ability of HIV vaccines to generate viral-specific CTL and because their development may contribute to protection, we undertook to compare two vaccine regimens, only one of which should be effective at generating CD8^{+} CTL, for their ability to generate gp160-specific CTL in seronegative volunteers.

Both vaccination approaches have been found to be well tolerated (15,16). The gp160 subunit vaccine stimulated anti-gp160 antibodies in 91% of the vaccinees, as detected by Western blot, and neutralizing antibodies to the immunizing virus in 21% of the subjects (15). Furthermore a gp160-specific lymphoproliferative response was seen in three of five volunteers tested (27). Likewise, in a randomized, blinded, phase I study, Graham et al. reported that vaccination with HIVAC-1e elicited both antibodies and lymphoproliferative responses in 73 and 50% of the vaccinees, respectively (16). Boosting of the

HIVAC-1e recipients with subunit gp160 increased the antibody response to 100%, with 67% of the individuals showing neutralizing antibodies (14).

Several studies have looked at the cellular immune response to these vaccines in healthy human volunteers. Use of recombinant gp160 subunit elicited both humoral and helper T-cell immunity (15, 27–29). Cooney et al. have shown that vaccination of seronegative individuals with recombinant vaccinia expressing gp160 elicited an HIV-specific lymphoproliferative response (30). Boosting with subunit gp160 enhanced this response in addition to inducing a humoral response, both of which persisted beyond 12 months (31). The authors also demonstrated both a CD8^{+} and CD4^{+} CTL response to gp160 after in vitro stimulation. They did not report on HIV-specific CTL activity in vaccinees given subunit gp160 alone. Reconstitution of
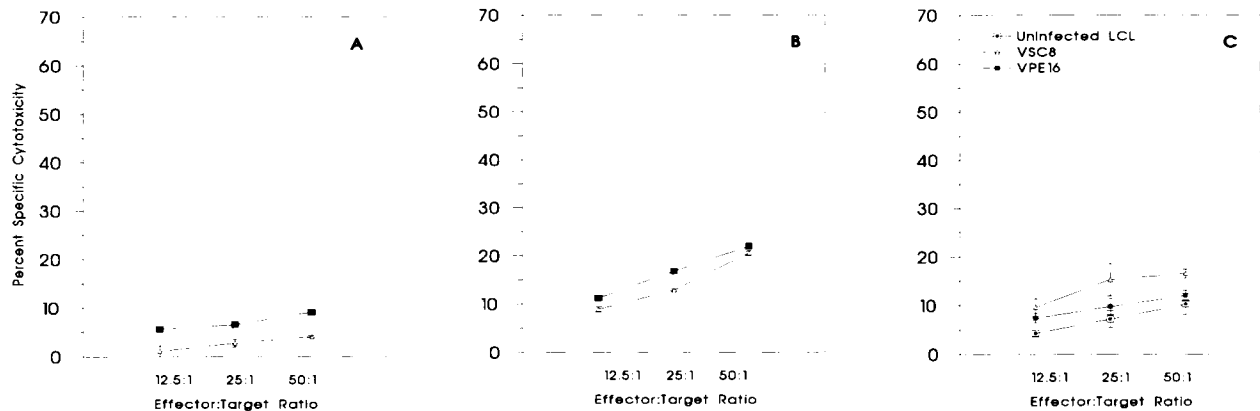

FIG. 4. CD4-enriched T cells from vaccinees immunized with subunit gp160 protein do not lyse HIV-1 gp160-bearing targets in a 4-h ^{51}Cr release assay. A, B, and C show results from CD8-immunomagnetic bead-depleted T cells obtained from cell lines generated four weeks after the fourth boost of subjects 003BF13, 003BF14, and 003BF15, respectively.

SCID mice with PBMCs from volunteers in the HIVAC-1e plus baculovirus gp160 study conferred protection against subsequent infection with HIV in this model (32). Hammond et al. (33) were also able to demonstrate HIV-specific CTL after in vitro stimulation in recipients of the combined HIVAC-1e-baculovirus gp160 regimen. Cloning revealed a predominance of CD8$^+$ clones with some HIV-specific CD4$^+$ clones (33). One group has also been able to demonstrate the presence of gp160-specific cytolytic CD4$^+$ clones in 3/8 volunteers immunized with rgp160 (34). Several studies have evaluated recombinant vaccinia virus expressing the envelope gene as a candidate vaccine in animals. Zarling et al. showed that PBL from vaccinia-env immunized chimpanzees were able to both proliferate and exhibit CTL capable of recognizing HIV envelope glycoprotein (35). However, when challenged with virus, no protection could be shown (36). More recently Hu et al. (26) have demonstrated protection against viral challenge in the SIV macaque model by immunization with recombinant vaccinia virus expressing SIVmne gp160 followed by boosting with subunit gp160. This regimen, comparable to one used in this study, induced neutralizing antibodies in addition to a lymphoproliferative response (26).

We have previously shown that the use of unselected T-cell lines is an effective method for the study of CTL in HIV-1 infected patients (37). In this study, we have used this method to show modest gp160-specific CTL in several individuals immunized with HIVAC-1e and boosted with subunit gp160. This response was further shown in one subject to be mediated by both CD8$^+$ and CD4$^+$ T lymphocytes and to persist for at least four months after boosting. Individuals vaccinated with subunit gp160 alone, however, displayed no detectable *env*-specific cytotoxicity.

In one immunized subject we found comparable CD4- and CD8-mediated gp160-specific cytotoxicity. Our finding of gp160-specific CD4$^+$ CTL is consistent with the finding of gp160-specific CD4$^+$ CTL clones after in vitro selection in vaccinia plus boost immunized patients by Cooney et al. (31) and Hammond et al. (33). BALB/c mice immunized with *env*-vaccinia also develop CD4$^+$ gp160-specific CTL, although the predominant CTL response is CD8-mediated (38). In the future it will be interesting to compare subunit immunization with live vector immunization for their relative ability to induce CD4$^+$ CTL as well as other aspects of CD4$^+$ T cell function including secretion of cytokines.

We studied the gp160-specific cytotoxicity of T-cell lines derived from one volunteer immunized only with adjuvant and from five unimmunized volunteers. Although the unimmunized volunteers demonstrated no gp160-specific activity, the adjuvant control T-cell line displayed modest amounts of specific cytotoxicity, comparable to that of some of the vaccinees in the vaccinia plus boost arm. There have been reports of gp160-specific CTL in some seronegative (and presumably unexposed) subjects detectable after in vitro stimulation. The prevalence of this is unclear. In one study, Hoffenbach et al. described a high-frequency of gp160-specific precursor CTL in four of six HIV-seronegative subjects (6). The finding of gp160-specific CTL activity in the line from the adjuvant only control subject together with the low levels of cytotoxicity we have observed in unselected T-cell lines suggests that our results be interpreted with caution. Future studies of cytotoxicity in immunized subjects will need to confirm these results. Our results can be improved in future studies by in vitro enhancement of immunogen-specific cytotoxicity using antigen-presenting cells transfected or infected with nonvaccinia vectors, such as avipox. The limited samples available to us did not enable us to explore other methods of in vitro selection on these subject samples. In subjects 002A52 and 002A54, however, we were able to look at gp160-specific cytotoxicity for each subject with preimmune cells and with cells obtained at four separate times after immunization. Our finding of significant, albeit modest, cytotoxicity at all postimmunization, but not preimmunization, timepoints supports our finding that the measured gp160-specific cytotoxicity is the result of the immunization.

Other studies of the CTL response to HIV vaccines cited above have relied on in vitro stimulation with antigen to demonstrate CTL activity. This study is the first evidence of gp160-specific CTL without any specific in vitro selection. That specific CTL can be demonstrated even without in vitro selection suggests that the HIVAC-1e immunization generates a potent CTL response. We were unable to enhance this cytotoxicity by in vitro stimulation with vPE16-infected APCs because the patient secondary in vitro response to vaccinia overshadowed any secondary in vitro response to gp160 (data not shown).

This study is also the first direct comparison of the CTL response elicited by two HIV vaccines using the same in vitro expansion protocol. Our re-

sults confirm the expectation that a live vector can be used to generate CD8 CTL immunity, but that whole protein subunit vaccines are either unable to generate specific CTL or generate a significantly weaker response falling below our level of detection. As demonstrated by FACS analysis, our culture conditions led to a preferential expansion of CD8$^+$ T cells. Because a protein subunit vaccine would be expected to induce a predominantly CD4$^+$ response, which might be masked by a greater number of CD8$^+$ cells, the T-cell lines were also depleted of CD8$^+$ cells and tested for cytotoxicity. No specific cytolytic response was detected under these conditions either. However, we cannot exclude the possibility that this whole-protein vaccine is, in fact, able to elicit a weak HIV-specific CTL response in some individuals that is only detectable after specific in vitro stimulation. The fact that we were able to demonstrate a response in the vaccinia-treated group using nonselected lines suggests that the CTL precursor frequency is higher with live virus vaccination.

Confirming results from other studies, the CTL responses in individuals vaccinated with HIVAC-1e and boosted with subunit gp160 were variable and in some cases quite low. Graham et al. showed that the vaccinia vector was genetically stable in its expression of gp160 after inoculation of volunteers (16). It is therefore likely that host factors, including expected differences in the cellular immune response to a given antigen in an MHC-diverse population, are the main reason for the observed variability. An important element with implications for large-scale immunization trials is whether or not vaccinated individuals have previously been primed with vaccinia. Previous studies have shown that immunization with vaccinia recombinants is more effective in eliciting antibody and lymphoproliferative responses in vaccinia-naive individuals (31–33). However, all of the HIVAC-1e recipients in this study were vaccinia-naive, so no conclusions can be drawn about this issue from this study. The ability of priming with a live vaccine, but not immunization with only subunit protein, to elicit detectable CD8$^+$ T-cell responses lends support to the current model for antigen presentation. Furthermore, although others have reported the presence of CD4$^+$ CTL after vaccination with the subunit protein vaccine, no CD8$^+$ response has been published, in agreement with the antigen processing model.

It is not known from animal studies what type of immunity should be elicited by a preventive vaccine to ensure its effectiveness. It seems likely that both neutralizing antibodies and cell-mediated immunity may play a significant role. Although most vaccine studies have focused on the development of neutralizing antibodies, little is known about the role, if any, of T-cell immunity in protection from new infection. In murine LCMV infection, Klavinskis et al. (9) have shown a purely CD8 T-cell response can be protective. In mice of H-2^d background, a 6-amino acid nucleoprotein peptide dominates the CTL response and a single immunization with a recombinant vaccinia vector encoding a truncated nucleoprotein confers complete protection against intracranial viral challenge (9). Protection against infection correlated with viral-specific CTL activity. The possible existence of HIV-seronegative high-risk subjects with demonstrable T-cell proliferative responses but no detectable specific antibodies to HIV suggests, however, that viral-specific T cells may also confer protective immunity in HIV-1 infection (39). The conditions for optimization of a cell-mediated immune response differ from those that maximize humoral immunity. Design of an ideal vaccine strategy will vary from pathogen to pathogen and, in the end, clinical trials in populations at risk for infection will be necessary to determine the relative protection conferred by cell-mediated immunity and humoral immunity and to develop an effective HIV vaccine.

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