

# Comparative Clonal Analysis of Human Immunodeficiency Virus Type 1 (HIV-1)-specific CD4<sup>+</sup> and CD8<sup>+</sup> Cytolytic T Lymphocytes Isolated from Seronegative Humans Immunized with Candidate HIV-1 Vaccines

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## Summary

The lysis of infected host cells by virus-specific cytolytic T lymphocytes (CTL) is an important factor in host resistance to viral infection. An optimal vaccine against human immunodeficiency virus type 1 (HIV-1) would elicit virus-specific CTL as well as neutralizing antibodies. The induction by a vaccine of HIV-1-specific CD8<sup>+</sup> CTL in humans has not been previously reported. In this study, CTL responses were evaluated in HIV-1-seronegative human volunteers participating in a phase I acquired immune deficiency syndrome (AIDS) vaccine trial involving a novel vaccine regimen. Volunteers received an initial immunization with a live recombinant vaccinia virus vector carrying the HIV-1 *env* gene and a subsequent boost with purified *env* protein. An exceptionally strong *env*-specific CTL response was detected in one of two vaccine recipients, while modest but significant *env*-specific CTL activity was present in the second vaccinee. Cloning of the responding CTL gave both CD4<sup>+</sup> and CD8<sup>+</sup> *env*-specific CTL clones, permitting a detailed comparison of critical functional properties of these two types of CTL. In particular, the potential antiviral effects of these CTL were evaluated in an in vitro system involving HIV-1 infection of cultures of normal autologous CD4<sup>+</sup> lymphoblasts. At extremely low effector-to-target ratios, vaccine-induced CD8<sup>+</sup> CTL clones lysed productively infected cells present within these cultures. When tested for lytic activity against target cells expressing the HIV-1 *env* gene, CD8<sup>+</sup> CTL were 3–10-fold more active on a per cell basis than CD4<sup>+</sup> CTL. However, when tested against autologous CD4<sup>+</sup> lymphoblasts acutely infected with HIV-1, CD4<sup>+</sup> clones lysed a much higher fraction of the target cell population than did CD8<sup>+</sup> CTL. CD4<sup>+</sup> CTL were shown to recognize not only the infected cells within these acutely infected cultures but also noninfected CD4<sup>+</sup> T cells that had passively taken up gp120 shed from infected cells and/or free virions. These results were confirmed in studies in which CD4<sup>+</sup> lymphoblasts were exposed to recombinant gp120 and used as targets for gp120-specific CD4<sup>+</sup> and CD8<sup>+</sup> CTL clones. gp120-pulsed, noninfected targets were lysed in an antigen-specific fashion by CD4<sup>+</sup> but not CD8<sup>+</sup> CTL clones. Taken together, these observations demonstrate that in an in vitro HIV-1 infection, sufficient amounts of gp120 antigen are produced and shed by infected cells to enable uptake by cells that are not yet infected, resulting in the lysis of these noninfected cells by gp120-specific, CD4<sup>+</sup> CTL. However, the antigen processing pathways involved in this reaction do not allow for association of gp120 peptides with class I molecules on the target cells. Thus, in contrast to CD4<sup>+</sup> CTL, gp120-specific CD8<sup>+</sup> CTL can correctly distinguish between productively infected cells and noninfected T cells that have acquired gp120 via CD4. With respect to the critical question of crossreactivity of vaccine-induced CTL on divergent strains of HIV-1, some vaccine-induced CD8<sup>+</sup> CTL clones showed complete crossreactivity with targets expressing the *env* genes of the divergent MN and RF isolates. The implications of these findings for the development of HIV-1 vaccines and for the pathogenesis of AIDS are discussed.

CTL can control the spread of viral infections within the host by eradicating infected host cells (1–3). In the case of HIV-1 infection, a vigorous HIV-1-specific, CD8<sup>+</sup> CTL response is detectable in the majority of seropositive individuals (4–7). Activated CD8<sup>+</sup> T cells from such individuals can suppress HIV-1 replication *in vitro* through direct lysis of infected cells and/or cytokine-mediated inhibition of viral replication (8–10). Although HIV-1-specific CD8<sup>+</sup> CTL ultimately fail to control HIV-1 infection *in vivo*, it is likely that the antiviral activity of these CTL helps to prolong the asymptomatic phase of infection (11).

In the vaccine setting, HIV-1-specific CTL induced by immunization could potentially eliminate cells initially infected by HIV-1 and prevent the establishment of disseminated chronic infection. Thus, there has been a great deal of interest in the design of vaccines capable of eliciting HIV-1-specific CTL. Virus-specific CTL are typically derived from the CD8<sup>+</sup> T cell subset and recognize processed peptide fragments of viral antigen in association with class I MHC gene products expressed on the surface of an infected cell (12, 13). As a general rule, the processing of a viral protein for association with class I MHC gene products and subsequent recognition by CD8<sup>+</sup> CTL occurs only when the viral protein is synthesized within the infected cell. In contrast, viral proteins taken up from the extracellular environment by an endocytic pathway are generally processed for association with class II MHC gene products and subsequent recognition by CD4<sup>+</sup> T cells (14–17). It is therefore not surprising that immunization of human volunteers with purified forms of the HIV-1 envelope protein precursor gp160 elicits a T cell response that consists exclusively of CD4<sup>+</sup> T cells. Interestingly, in some gp160 recipients, a majority of the responding *env*-specific CD4<sup>+</sup> T cell clones have cytolytic activity (18). The induction by a vaccine of a CD8<sup>+</sup> CTL response specific for HIV-1 has not been previously described in humans. Current notions about antigen processing suggest that the induction of such a response would require vaccination with live virus vectors such as recombinant vaccinia constructs carrying HIV-1 genes (19, 20), or the use of other novel vaccine strategies that permit delivery of viral proteins to the cytoplasm of host cells (21–23). In animal models, recombinant vaccinia virus vectors have been successfully used to elicit CTL responses against retroviral antigens (24–28).

In a recent phase I vaccine trial, a small number of HIV-1-seronegative human volunteers were immunized with a live recombinant vaccinia virus vector carrying the HIV-1 *env* gene (*vac-env*) and boosted with purified recombinant gp160. This combined vaccine regimen is modeled after a similar vaccine strategy that has been effective in the simian immunodeficiency virus system (29). With respect to the induction of CD8<sup>+</sup> CTL, this combined regimen is of particular interest because of the potential of *vac-env* infection to elicit CD8<sup>+</sup> CTL specific for the HIV-1 envelope protein. In addition, *env*-specific CD4<sup>+</sup> T cells activated by the initial *vac-env* immunization and/or by the gp160 boost may enhance the clonal expansion of memory *env*-specific CD8<sup>+</sup> CTL upon subsequent exposure to HIV-1-infected cells.

The present study describes CTL responses in HIV-1-

seronegative human volunteers immunized with this combined *vac-env*/gp160 vaccine regimen and provides the first demonstration that HIV-1-specific CD8<sup>+</sup> CTL can be induced in humans by candidate HIV-1 vaccines. The responding CTL were cloned, and both CD4<sup>+</sup> and CD8<sup>+</sup> *env*-specific clones were obtained, permitting a detailed comparative analysis of the interactions of these two different classes of CTL with HIV-1-infected cells and with noninfected CD4<sup>+</sup> T cells that have taken up free envelope protein via CD4. The results of these studies demonstrate the value of vaccine-induced CD8<sup>+</sup> CTL in the destruction of infected cells, but heighten concern over the potential of CD4<sup>+</sup> CTL to contribute to CD4<sup>+</sup> T cell depletion *in vivo* through the destruction of noninfected cells.

## Materials and Methods

**Vaccine Trial.** Vaccinia-naive HIV-1-seronegative volunteers were immunized with candidate HIV-1 vaccines in a phase I vaccine trial organized by the NIAID AIDS Vaccine Clinical Trials Network. The vaccine regimen involved an initial immunization with a live *vac-env* vector (HIVAC-1e; Oncogen/Bristol-Myers Squibb, Seattle, WA), which carries the *env* gene of the reference LAI isolate of HIV-1. The preparation of this vector and initial studies of its safety and immunogenicity have been presented in detail elsewhere (19, 30, 31). The *vac-env* vector was administered by standard intradermal scarification with bifurcated needle punctures. Some volunteers were immunized with a control vaccinia vector lacking the HIV-1 *env* gene. 2 mo after immunization, *vac-env* recipients were boosted with 640 µg recombinant HIV-1 (LAI) gp160 (VaxSyn; MicroGeneSys, Inc., Meriden, CT). Previous studies have described the safety and immunogenicity of recombinant gp160 in HIV-1-seronegative (32) and healthy HIV-1-seropositive (33) volunteers. The design of this combined *vac-env*/gp160 regimen and initial results of studies of the safety and immunogenicity of this regimen are described in detail elsewhere (B. Graham et al., manuscript submitted for publication; P. Greenberg et al., manuscript submitted for publication).

**Antigens.** Purified recombinant HIV-1 (LAI) gp160 and gp120 produced in the baculovirus expression system were obtained from MicroGeneSys, Inc.

**Constructs and Transfections.** Autologous EBV-transformed B lymphoblastoid cell lines (B-LCL)<sup>1</sup> from vaccinees and controls were transfected by electroporation with an EBV-based eukaryotic expression vector (34) carrying the HIV-1 *env* gene (pCEP4*env*). The plasmid pCEP4*env* was constructed by inserting into the multiple cloning site of pCEP4 (Invitrogen, San Diego, CA), the *SalI*-*XhoI* fragment of the pIII*env*3-1 (35), a plasmid containing the HIV-1 (LAI) *env* gene. The pIII*env*3-1 plasmid was obtained from the AIDS Research and Reference Reagent Program (Rockville, MD) (contributor, Dr. Joseph Sodroski). In addition to the *env* coding sequence, this fragment contains both exons of the *rev* gene, expression of which is necessary for the production of *env* protein in this system. In pCEP4*env*, both *env* and *rev* are under the control of the constitutive cytomegalovirus immediate-early promoter. Each transfected B-LCL was placed into selective media containing appropriate predetermined concentrations of hygromycin B for ≥6 d before use in the *in vitro* stimulation of *env*-specific memory T cells. Expression of the HIV-1 *env* gene by transfectants was verified by indirect immunofluorescence using the mAb 902. This anti-

<sup>1</sup> Abbreviation used in this paper: B-LCL, B lymphoblastoid cell line.

body, kindly provided by Drs. Jonathan Yewdell and Bruce Chesebro (National Institutes of Health), recognizes gp120 expressed on the surface of infected cells (36).

**In Vitro Stimulation of *env*-specific CTL.** 1 mo after the gp160 boost, PBMC were isolated from vaccinees and controls by discontinuous density gradient centrifugation and were stimulated in vitro with *env*-transfected B-LCL. Stimulations were carried out by culturing PBMC with lethally irradiated (7,000 rad), *env*-expressing autologous B-LCL in IL-2-free culture medium (CM) consisting of RPMI 1640, 10% FCS, 4 mM glutamine, 50 U/ml penicillin, and 50 µg/ml streptomycin. On day 4 of the in vitro stimulation, the media was supplemented with 50 U/ml of IL-2. Cytolytic activity was measured 7–9 d after initiation of the cultures.

**Functional Assays.** Cytotoxicity was measured in standard <sup>51</sup>Cr-release assays at the indicated E/T ratios as previously described (37), except that the assay period was 8 h. Target cells were pulsed with the indicated recombinant antigens, or infected with HIV-1 or recombinant vaccinia viruses, as described below, before labeling with <sup>51</sup>Cr and use in the cytolytic assay. The SEM of the percent specific lysis, determined as previously described (38), was almost always <5%.

**Vaccinia Vectors.** The use of vaccinia virus vectors for the expression of the HIV-1 *env* gene has been described (19, 20). In the present study, the vectors vPE16 and vPE8, which contain the *env* gene of LAI isolate (BH8 clone) of HIV-1, were used. vPE16 carries the full-length *env* gene modified to remove vaccinia virus transcription termination sequences (39). vPE8 carries a truncated form of the *env* gene with a stop codon at the end of the gp120 coding sequence (40). Vectors carrying the *env* genes of the RF and MN strains have been previously described (6). Control infections were carried out with the vaccinia vector vSC8, which lacks the HIV-1 *env* gene, or with the standard wild-type WR vaccinia strain used in the preparation of vaccinia recombinants (41). Target cells were infected with recombinant vaccinia viruses at a multiplicity of infection of 10 for 12–18 h at 37°C before labeling with <sup>51</sup>Cr and use in cytolytic assays. Vaccinia vectors used in this study were kindly provided by Drs. Pat Earl and Bernard Moss of the National Institutes of Health and by the AIDS Research and Reference Reagent Bank.

**Isolation of T Cell Clones.** On day 13 after a single in vitro stimulation with *env*-transfected autologous B-LCL, cultures with *env*-specific cytolytic activity were cloned by limiting dilution using PHA (0.25 µg/ml; Wellcome Diagnostics, Research Triangle Park, NC) and irradiated allogeneic PBMC as previously described (18). To assure a high (>95%) probability of monoclonality, cells were isolated only from plates seeded with doses of responding cells for which <10% of the wells were positive for growth. Clones were screened for HIV-1 *env*-specific cytolytic activity in <sup>51</sup>Cr-release assays with mock-infected, vSC8-infected, and vPE16-infected autologous B-LCL targets. All T cell clones and lines were maintained in the presence of IL-2 and were restimulated every 7–10 d. For restimulation, clones were cultured in 24-well plates at 5 × 10<sup>5</sup>/ml with irradiated (5,000 rad) allogeneic PBMC and PHA. 3 d after restimulation, the media were replaced with fresh CM containing IL-2 at 150 U/ml for CD8<sup>+</sup> clones or at 50 U/ml for CD4<sup>+</sup> clones. All clones were given two-part designations (e.g., A42.45), in which the first part indicates the volunteer identification number and the second part indicates the individual clone from that volunteer. A single set of volunteer identification numbers is used in all publications describing results from HIV-1 vaccine trials organized by the NIAID AIDS Vaccine Clinical Trials Network.

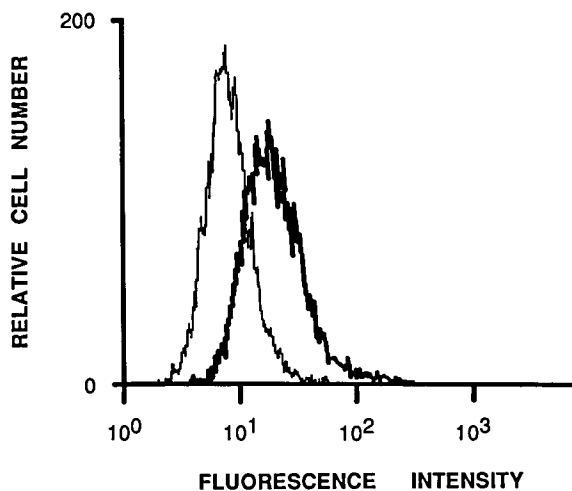
**HIV-1 Infections.** To generate autologous CD4<sup>+</sup> target cell lines, PBMC from volunteers were stimulated with PHA and sub-

sequently subjected to two cycles of cell sorting for CD4 expression. The resulting polyclonal T cell lines were >99% CD4<sup>+</sup>. The lines were maintained by weekly restimulation in IL-2-containing medium with PHA and irradiated allogeneic PMBC as described above. The phenotype was checked biweekly and remained exclusively CD4<sup>+</sup>. The CD4<sup>+</sup> T cell lines were mock infected or infected with HIV-1 3 d after restimulation with PHA. Infections were carried out by resuspending the CD4<sup>+</sup> T cells in cell-free supernatants of SupT1 cells infected 7 d previously with HIV-1 (LAI isolate) as previously described (18). After 4 h at 37°C, CD4<sup>+</sup> T cells were washed and then cultured in IL-2-containing media. Infected cells were used as targets in <sup>51</sup>Cr-release assays 4–7 d after exposure to HIV-1. The percent of infected cells in these cultures was assessed by two different methods with comparable results. The first method involved staining of permeabilized cells for HIV-1 *gag* antigen and has been previously described (18). The second method involved staining of the infected cell culture with an anti-CD4 mAb (12T4D11; Coulter Immunology, Hialeah, FL) that recognizes an epitope on CD4 that is spatially distinct from the gp120 binding site. Because of the complexing of CD4 with gp160 in the endoplasmic reticulum, cells productively infected with HIV-1 show a dramatic decrease in surface expression of CD4, and are readily distinguishable from noninfected cells that have taken up free gp120 via CD4 (42, 43).

## Results

**Analysis of CTL Responses in Vaccine Recipients.** To evaluate the human CTL response induced by the *vac-env/gp160* regimen, an in vitro stimulation was used to activate and expand *env*-specific memory T cells in peripheral blood from vaccinees. Autologous B-LCL that had been transfected with the HIV-1 *env* gene were lethally irradiated and used to stimulate PBMC from vaccine recipients and controls. Like the macrophages and activated CD4<sup>+</sup> T cells that are productively infected with HIV-1 in natural infection, B-LCL express both class I and class II MHC molecules. Since the HIV-1 envelope protein synthesized in infected cells is processed for association with both class I (4–7) and class II (18, 44) MHC molecules, these transfectants should stimulate *env*-specific memory T cells from both the CD8<sup>+</sup> and the CD4<sup>+</sup> subsets. Fig. 1 shows results of an indirect immunofluorescence assay demonstrating surface expression of the HIV-1 *env* protein by *env*-transfected B-LCL. Expression of the *env* gene was confirmed by immunoprecipitation of *env* protein (data not shown).

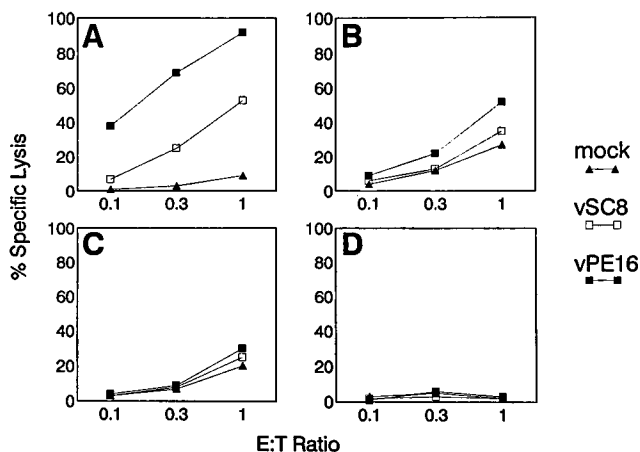
9 d after stimulation with *env* transfectants, responding T cells were assayed for *env*-specific cytolytic activity. As shown in Fig. 2 A, stimulated PBMC from *vac-env/gp160* recipient no. A42 showed significant *env*-specific cytolytic activity that was 30–45% above the background level of vaccinia-specific cytolysis. Notably, this culture showed *env*-specific lytic activity even at low E/T ratios (0.1:1). Stimulated PBMC from a second *vac-env/gp160* recipient (no. A52) also showed modest but significant *env*-specific CTL activity (Fig. 2 B). In contrast, a control vaccinee (no. A41) immunized with wild-type vaccinia did not have detectable *env*-specific CTL activity (Fig. 2 C). Stimulated PBMC from nonimmunized, HIV-1-seronegative volunteers ( $n = 2$ ) were also negative for *env*-specific CTL activity (Fig. 2 D). These results demonstrate



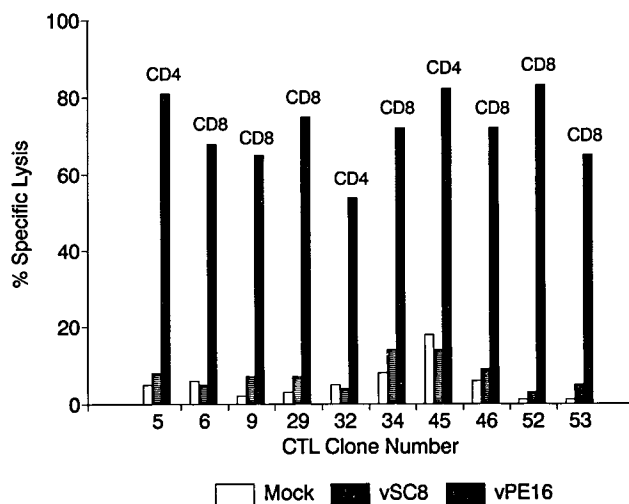
**Figure 1.** Expression of the HIV-1 envelope protein by stimulator cells used to activate memory CTL in vaccine recipients. B-LCL were transfected with the HIV-1 *env* gene as described in Materials and Methods. Expression of envelope protein on *env*-transfected (thick line) and control untransfected (thin line) B-LCL was assessed by indirect immunofluorescence using the anti-gp120 mouse mAb 902. Background staining of transfected and untransfected cells with an irrelevant primary antibody was identical.

that the *vac-env*/gp160 regimen can induce *env*-specific CTL in seronegative humans.

**Cloning of *env*-specific CTL.** To characterize in a definitive fashion the *env*-specific CTL induced by this vaccine regimen, limiting dilution cloning of the culture from volun-



**Figure 2.** HIV-1 *env*-specific cytolytic activity in stimulated cultures from vaccine recipients and controls. 9 d after initial in vitro stimulation of PBMC from vaccine recipients with autologous *env*-transfected B-LCL, responding cells were assayed for cytolytic activity against autologous B-LCL that had been either mock infected, infected with a control vaccinia virus vector (vSC8), or infected with a *vac-env* vector (vPE16). Effector cells were: (A) stimulated PBMC from the vaccinia-naive, HIV-1-seronegative *vac-env*/gp160 recipient no. A42; (B) stimulated PBMC from the vaccinia-naive, HIV-1-seronegative *vac-env*/gp160 recipient no. A52; (C) stimulated PBMC from a vaccinia-naive, HIV-1-seronegative volunteer (no. A41) immunized with parental wild-type vaccinia; or (D) stimulated PBMC from a representative nonimmune, HIV-1-seronegative donor.



**Figure 3.** Specificity and phenotype of representative CTL clones from *vac-env* recipient no. A42. Clones were assayed for cytolytic activity against autologous B-LCL that had been either mock infected (Mock), infected with a control vaccinia vector (vSC8), or infected with a *vac-env* vector (vPE16). The phenotype of each CTL clone, as determined by immunofluorescence with anti-CD4 and anti-CD8 mouse mAbs, is indicated. Clones were tested at E/T ratios giving optimal lysis (1:1 for CD8<sup>+</sup> CTL clones, and 30:1 for CD4<sup>+</sup> CTL clones).

**Table 1.** MHC Restriction and Epitope Specificity of Vaccine-induced CTL Clones

Clone	MHC restriction*	Epitope localization†
A42.6	NT <sup>§</sup>	gp120
A42.9	A3.1	gp120
A42.29	A3.1	gp120
A42.34	NT	gp120
A42.46	A3.1	gp120
A42.52	B35	gp41
A42.53	NT	gp120
A52.161	A3.1	gp120

\* Clones were tested for lytic activity against control vaccinia-infected and *vac-env*-infected target cells that were either MHC mismatched or that shared a single class I allele with the donor. Donor genotypes were as follows. Donor no. A42: A3, A11, B18, B35, Bw4, Bw6, Cw4, Cw5, DR3, DRw6(13), DQw1, DQw2; donor no. A52: A1, A3, B8, B35, Bw6, Cw4, DR1, DR5 (DRw12), DQw1, DQw3. For each clone tested, only targets sharing the indicated allele were lysed in an antigen-specific fashion.

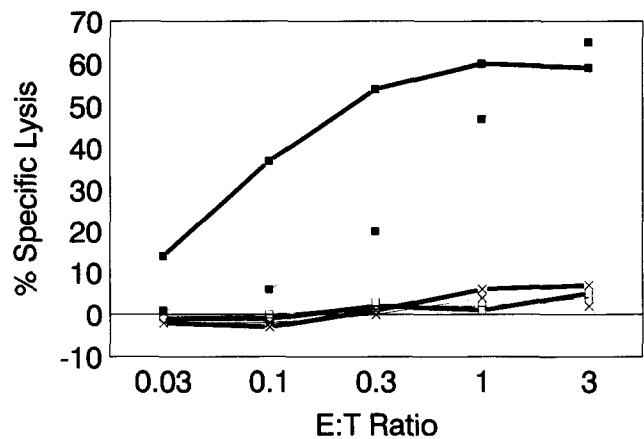
† Epitopes recognized by *env*-specific CTL clones were localized to the gp120 or gp41 subunits of the envelope protein using CTL assays with autologous B-LCL targets infected with the vaccinia vectors vPE16, which carries the full-length *env* gene, and vPE8, which carries a truncated form of the *env* gene with a stop codon at the end of the gp120 coding sequence. See Materials and Methods.

§ NT, not tested.

teer no. A42 was carried out after a single in vitro stimulation of PBMC with *env*-transfected autologous B-LCL. Flow cytometric analysis revealed that >90% of the responding T cells in this culture were CD8<sup>+</sup>. The cloning was carried out under conditions that have been previously shown to permit efficient clonal expansion of all CD4<sup>+</sup> and CD8<sup>+</sup> human T cells (45) so that the clones obtained would reflect the composition of the culture at the time of cloning. Clones that grew out under these conditions were screened for *env*-specific cytolytic activity. Of the clones screened, a remarkable 36% (20/56) were specific for *env* antigen. Of these, 10 clones grew well enough to permit detailed analysis of their functional properties. The majority of the clones obtained were CD8<sup>+</sup> and showed a high level of lytic activity against target cells expressing the HIV-1 *env* gene (Fig. 3). However, CD4<sup>+</sup> CTL clones specific for the HIV-1 *env* protein were also obtained. Thus, the *vac-env/gp160* regimen induced *env*-specific CTL from both T cell subsets. *env*-specific CD8<sup>+</sup> CTL were also cloned from *vac-env/gp160* recipient no. A52.

Extensive previous analysis of *env*-specific CD4<sup>+</sup> CTL cloned from HIV-1 vaccine recipients has shown that these CTL show classic MHC restriction (18, 37, 46). To determine whether the CD8<sup>+</sup> CTL described here were MHC restricted, vaccine-induced clones were tested for lytic activity against control vaccinia-infected and *vac-env*-infected target cells that were either MHC mismatched or that shared a single class I allele with the donor (Table 1). These studies demonstrated that each of the CD8<sup>+</sup> clones tested showed classic MHC restriction to a single self class I allele. In addition, B-LCL targets infected with recombinant vaccinia vectors carrying wild-type or truncated forms of the HIV-1 *env* gene were used to determine whether these clones recognized epitopes in the gp120 or gp41 subunits of the envelope protein. All of the clones were found to recognize epitopes in gp120, except for clone A42.52, which recognizes an epitope in gp41 (Table 1).

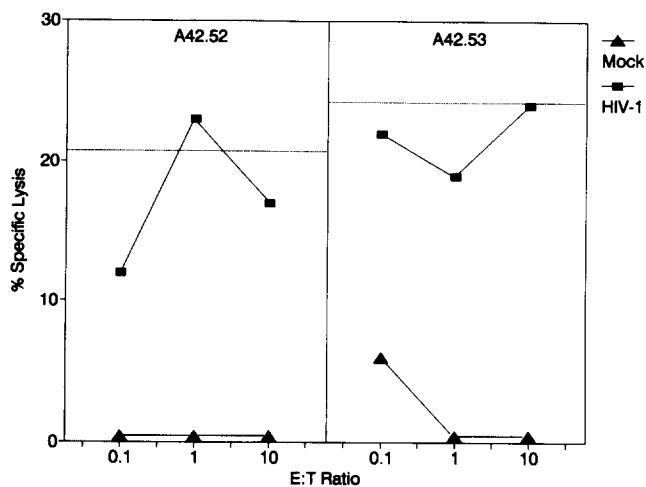
**Comparative Analysis of the Lytic Activity of CD8<sup>+</sup> and CD4<sup>+</sup> CTL Clones.** Because the HIV-1 envelope proteins are processed in infected cells for association with both class I and class II MHC gene products, it was possible to compare directly the lytic activity of vaccine-induced CD8<sup>+</sup> and CD4<sup>+</sup> CTL clones against the the same *env*-expressing B-LCL targets. Lytic activity was compared on a per cell basis by testing clones against *vac-env*-infected targets over a wide range of E/T ratios at a constant and optimal assay time of 8 h. Preliminary studies established that the kinetics of target cell lysis by both classes of CTL were similar (E. P. Miskovsky, P. E. Stanhope, W. Pavlat, and R. F. Siliciano, manuscript in preparation). Results from a representative experiment are shown in Fig. 4. Antigen-specific lysis was detected at extremely low E/T ratios (0.03:1) for the CD8<sup>+</sup> clone A42.46, while the CD4<sup>+</sup> clone A42.45 was inactive at this ratio. On a per cell basis, the CD8<sup>+</sup> CTL clone was 3–10-fold more efficient than the CD4<sup>+</sup> clone. Neither clone lysed mock-infected targets or target cells infected with control vaccinia vectors at any E/T ratio. Similar results were obtained with other vaccine-induced CD4<sup>+</sup> and CD8<sup>+</sup>



**Figure 4.** Comparative analysis of cytolytic activity of *env*-specific CD4<sup>+</sup> and CD8<sup>+</sup> CTL clones. Lytic activity of the representative *env*-specific CD4<sup>+</sup> CTL clone A42.45 (thin line) was compared with that of the representative *env*-specific CD8<sup>+</sup> CTL clone A42.46 (thick line) over a wide range of E/T ratios. Target cells were autologous B-LCL that had been either mock infected (×), or infected with a control vaccinia virus vector, vSC8 (□), or with the *vac-env* vector, vPE16 (■).

CTL clones with different epitope specificities and MHC restriction patterns, indicating that the more efficient lysis of target cells by CD8<sup>+</sup> CTL is a general property of this subset of T cells.

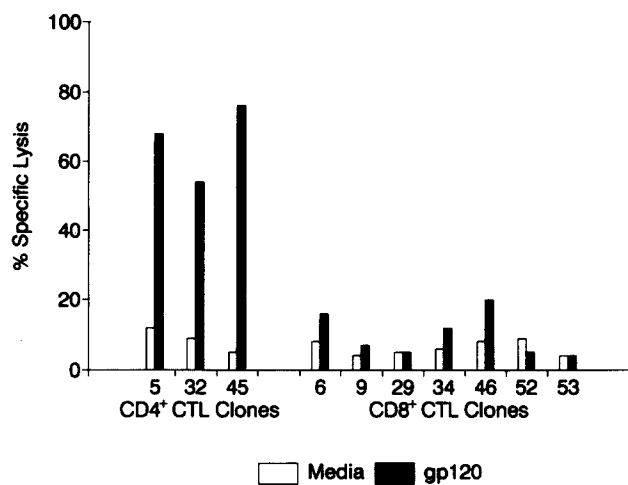
**Direct Lysis of HIV-1-infected Autologous T Cells by Vaccine-induced CD8<sup>+</sup> CTL.** The results presented above indicate that vaccine-induced, *env*-specific CD8<sup>+</sup> CTL have a very high level of cytolytic activity against *vac-env*-infected B-LCL targets, while CD4<sup>+</sup> CTL are somewhat less potent. However, with respect to vaccine efficacy, the critical question is whether vaccine-induced CTL lyse autologous cells infected with HIV-1. Analysis of this question is hampered by the difficulty in obtaining uniformly infected target cell populations of the appropriate MHC genotype. For these studies, target cells were prepared by infecting an autologous PHA-induced polyclonal CD4<sup>+</sup> T cell line with HIV-1 (LAI). 4–7 d later, infected cultures were used as a source of target cells in cytolytic assays with CD8<sup>+</sup> CTL clones. At this time, virus-induced cytopathic effects were generally not evident in the infected cultures, although 20–30% of the cells were productively infected as assessed by immunofluorescence. For two different vaccine-induced CD8<sup>+</sup> CTL clones, the percent of the target cells lysed reached a plateau with increasing E/T ratios, the plateau being almost exactly equal to the percentage of productively infected cells within the culture as determined by immunofluorescence analysis (Fig. 5). Plateau levels of lysis were reached at very low E/T ratios, indicating efficient antigen-specific lysis of HIV-1-infected autologous T cells, consistent with the efficient lysis of *vac-env*-infected targets described above. Lysis of infected cells was observed for both the gp41-specific CTL clone A42.52 and gp120-specific clone A42.53. Control mock-infected CD4<sup>+</sup> T lymphoblasts were not lysed by either clone. Taken together, these results demonstrate that vaccine-induced CD8<sup>+</sup> CTL specific for the envelope protein can efficiently lyse HIV-1-infected autologous T cells.



**Figure 5.** Lysis of HIV-1-infected, autologous CD4<sup>+</sup> T cells by vaccine-induced CD8<sup>+</sup> CTL clones. The gp41-specific CD8<sup>+</sup> CTL clone A42.52 and the gp120-specific CD8<sup>+</sup> CTL clone A42.53 were tested for lytic activity against autologous CD4<sup>+</sup> T lymphoblasts that had been either mock infected or infected with HIV-1 as described in Materials and Methods. For each experiment, the percent of the CD4<sup>+</sup> lymphoblasts that were productively infected as assessed by immunofluorescence analysis is indicated by the dotted horizontal line.

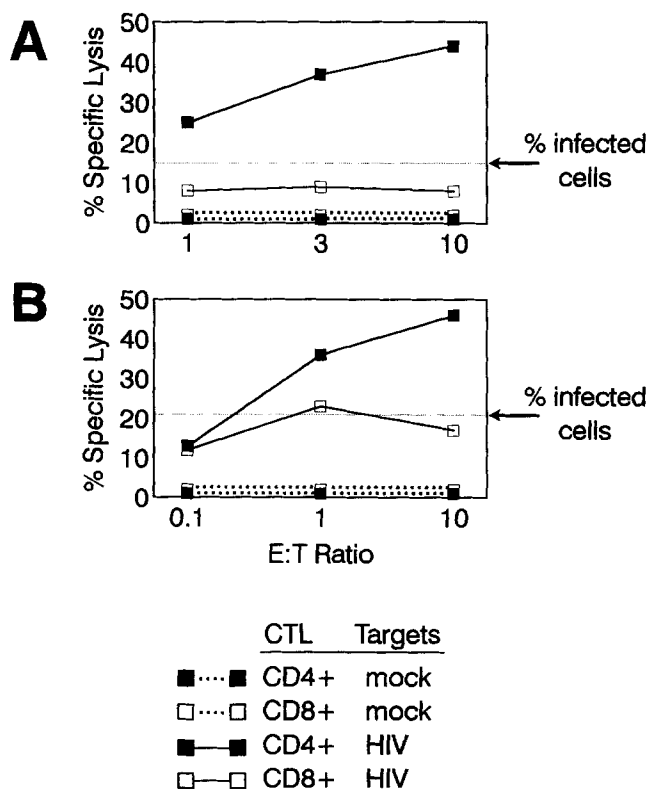
*CD4<sup>+</sup> but Not CD8<sup>+</sup> CTL Clones Mediate Lysis of Noninfected CD4<sup>+</sup> T Lymphoblasts Exposed to HIV-1 gp120.* In the peripheral lymphoid organs of HIV-1-infected individuals, noninfected CD4<sup>+</sup> T cells are exposed not only to HIV-1 virions released by infected cells but also to free gp120 released from infected cells and HIV-1 virions after the spontaneous dissociation of gp120-gp41 complexes (47, 48). Thus, while a fraction of the CD4<sup>+</sup> cells present in the vicinity of an infected cell will become productively infected, other CD4<sup>+</sup> T cells may simply bind gp120. Previous studies have shown that noninfected CD4<sup>+</sup> lymphoblasts can take up free gp120 via CD4 and process it for presentation with class II MHC gene products (18, 37, 49). More importantly, these noninfected cells can then be lysed by gp120-specific CD4<sup>+</sup> CTL in a reaction that may contribute to CD4<sup>+</sup> T cell depletion in natural infection (18, 37). To determine whether CD8<sup>+</sup> CTL mediate the gp120-dependent lysis of noninfected CD4<sup>+</sup> T cells, a series of vaccine-induced CD8<sup>+</sup> CTL clones was tested for cytolytic activity against an autologous CD4<sup>+</sup> T cell line that has been pulsed with media alone or with recombinant gp120 (Fig. 6). The gp120-pulsed targets were readily lysed by three different gp120-specific CD4<sup>+</sup> CTL clones (A42.5, A42.32, and A42.45). In contrast, there was only minimal lysis of the same gp120-pulsed targets by seven different CD8<sup>+</sup> CTL clones from the same donor. Neither CD4<sup>+</sup> nor CD8<sup>+</sup> clones lysed unpulsed target cells. These results establish that among the vaccine-induced gp120-specific CTL, CD4<sup>+</sup> clones lyse noninfected CD4<sup>+</sup> T lymphoblasts that have taken up soluble gp120, while CD8<sup>+</sup> clones are unable to do so.

*Lysis of Infected and Noninfected CD4<sup>+</sup> T Cells in an In Vitro Model for HIV-1 Infection.* Additional experiments were



**Figure 6.** Autologous CD4<sup>+</sup> T cells pulsed with gp120 are lysed by CD4<sup>+</sup> but not CD8<sup>+</sup> *env*-specific CTL clones. A PHA-activated, autologous CD4<sup>+</sup> T cell line was pulsed at 37°C overnight with media alone or with 30 μg/ml of recombinant gp120 from the LAI isolate. Pulsed CD4<sup>+</sup> T cells were then washed and labeled with <sup>51</sup>Cr. Lysis by the indicated CD4<sup>+</sup> and CD8<sup>+</sup> CTL clones was measured in a standard <sup>51</sup>Cr-release assay at E/T ratios giving optimal lysis (1:1 for CD8<sup>+</sup> CTL, and 30:1 for CD4<sup>+</sup> CTL). All of these clones with the exception of A42.52 are specific for epitopes in gp120.

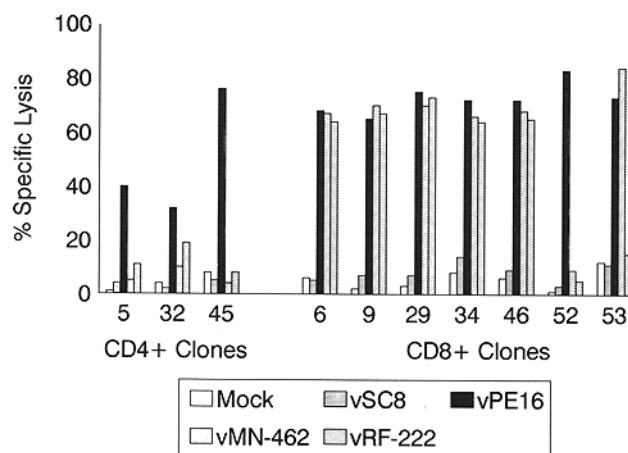
carried out to confirm the results presented above within the context of an in vitro HIV-1 infection. CD4<sup>+</sup> lymphoblasts were infected by exposure to supernatants from HIV-1-infected SupT1 cells. 4 d after infection, 10–30% of the cells showed evidence of productive infection by immunofluorescence analysis. However, 100% of these target cells stained for gp120 using an anti-gp120 mAb that detects endogenously synthesized gp120 as well as gp120 bound to CD4. Lysis of this acutely infected target cell population by representative vaccine-induced CD4<sup>+</sup> and CD8<sup>+</sup> CTL clones was analyzed (Fig. 7). CD8<sup>+</sup> CTL clones lysed a fraction of target cells equal to or slightly less than the number of productively infected cells in the culture as determined by immunofluorescence analysis. However, gp120-specific CD4<sup>+</sup> CTL clones consistently lysed a fraction of the target cells that was significantly higher than the fraction of productively infected cells within the culture. Although CD4<sup>+</sup> CTL can lyse HIV-1-infected cells (18), these data indicate that at least some of the target cells killed by CD4<sup>+</sup> CTL in this system were not productively infected. This lysis was antigen specific since no lysis was observed in the absence of HIV-1 infection. The higher lytic activity of CD4<sup>+</sup> CTL in this system is in sharp contrast to the results obtained in experiments with vac-*env*-infected target cells in which CD8<sup>+</sup> CTL were much more efficient on a per cell basis than CD4<sup>+</sup> CTL (Fig. 4). In the in vitro HIV-1 infection, the observed lysis by CD4<sup>+</sup> CTL of some cells that are not productively infected was consistent with flow cytometric evidence, demonstrating that a sufficient amount of gp120 antigen is produced and shed by infected cells in the culture to give detectable surface staining by all of the target cells. These data are also consistent with



**Figure 7.** Lytic activity of vaccine-induced CD4<sup>+</sup> and CD8<sup>+</sup> CTL against an acutely infected autologous CD4<sup>+</sup> T cell line. Autologous CD4<sup>+</sup> lymphoblasts were mock infected or were infected with HIV-1 by exposure to a cell-free supernatant from HIV-1-infected SupT1 cells. 4–7 d later, mock-infected and HIV-1-infected cultures were used as a source of target cells for cytolytic assays with the gp120-specific CD4<sup>+</sup> CTL clone A42.45 (A and B) and the CD8<sup>+</sup> CTL clones A42.53 (A) and A42.52 (B). For each experiment, the percent of the CD4<sup>+</sup> lymphoblasts that were productively infected as assessed by immunofluorescence analysis is indicated by the arrow.

the demonstration that CD4<sup>+</sup> lymphoblasts pulsed with recombinant gp120 are susceptible to lysis by vaccine-induced CD4<sup>+</sup> CTL but not by CD8<sup>+</sup> CTL (Fig. 6). Thus, in contrast to CD8<sup>+</sup> CTL, CD4<sup>+</sup> CTL specific for gp120 cannot correctly distinguish between productively infected cells and noninfected cells that have acquired gp120 in an in vitro HIV-1 infection.

**Crossreactivity of Vaccine-induced CD4<sup>+</sup> and CD8<sup>+</sup> CTL Clones on Diverse Isolates of HIV-1.** Striking sequence diversity is observed among HIV-1 isolates from different individuals (50–55), and to a lesser extent among sequential isolates and individual clones obtained from a given individual (56). A vaccine that induces anti-HIV-1 responses crossreactive with a spectrum of possible HIV-1 variants would be the most beneficial. Therefore, the crossreactivity of CTL clones induced by the *vac-env*/gp160 vaccine regimen on diverse HIV-1 isolates was assessed. Both the *vac-env* and the recombinant gp160 components of this vaccine regimen were derived from the reference LAI isolate of HIV-1. Autologous B-LCL were infected with recombinant vaccinia virus vectors expressing *env* genes from LAI or divergent strains of HIV-1, and were



**Figure 8.** Effect of HIV-1 sequence variability on CTL recognition of the HIV-1 *env* protein. The indicated vaccine-induced CTL clones were tested for lytic activity against mock-infected autologous B-LCL or autologous B-LCL infected with the control vaccinia vector (vSC8), or with vaccinia vectors carrying the *env* genes of the reference LAI isolate (vPE16), the MN isolate (vMN-462), or the RF isolate (vRF-222). Clones were tested at E/T ratios giving optimal antigen-specific lysis (1:1 for CD8<sup>+</sup> CTL, and 30:1 for CD4<sup>+</sup> CTL).

subsequently used as targets in a standard <sup>51</sup>Cr-release assay with vaccine-induced CTL clones (Fig. 8). The gp120-specific CD4<sup>+</sup> CTL clones A42.5, A42.32, and A42.45 all failed to recognize target cells expressing the *env* gene of the MN isolate, which may be more representative of HIV-1 strains prevalent in North America (57, 58). Clone A42.32 showed weak but consistent crossreactivity with the more highly divergent RF *env* protein, which was not recognized by clones A42.5 or A42.45. These results are consistent with previous studies, indicating that vaccine-induced CD4<sup>+</sup> CTL clones specific for determinants in gp120 display little crossreactivity with divergent HIV-1 isolates (18, 37, 59). In contrast, greater crossreactivity was detected with vaccine-induced CD8<sup>+</sup> CTL clones. Most importantly, several of the gp120-specific CD8<sup>+</sup> CTL clones recognized a highly conserved epitope, as evidenced by efficient lysis of cells expressing the *env* gene from the MN isolate and from the more highly divergent RF isolate. The epitope recognized by clone A42.46 is located in a highly conserved region at the NH<sub>2</sub> terminus of gp120 (P. Johnson, S. A. Hammond, R. F. Siliciano, and B. Walker, unpublished results). Interestingly, although the level of sequence variability in gp41 is considerably lower than that observed in the gp120 coding sequence, the gp41-specific CD8<sup>+</sup> CTL clone A42.52 failed to recognize either the MN or RF *env* protein. The gp120-specific CD8<sup>+</sup> CTL clone A42.53 lysed cells expressing the *env* protein from the MN isolate, but not from the RF isolate. Taken together, these results demonstrate that this vaccine regimen developed using the LAI isolate can induce CD8<sup>+</sup> CTL that recognize diverse isolates of HIV-1, including the representative North American isolate, MN, and, for some clones, even the more highly divergent RF isolate.

## Discussion

Studies presented here demonstrate that CD8<sup>+</sup> CTL specific for HIV-1 can be induced in HIV-1-seronegative humans by vaccination. A novel vaccine protocol involving immunization with a live *vac-env* vector followed by boosting with recombinant envelope protein was used. The present study extends important observations in murine and primate systems showing that immunization with live recombinant vaccinia vectors carrying retroviral genes can induce retrovirus-specific CD8<sup>+</sup> CTL (24–28), as well as earlier work in human volunteers showing that CD4<sup>+</sup> CTL can be induced by immunization with recombinant gp160 (18). Cloning studies described here establish definitively that both CD4<sup>+</sup> and CD8<sup>+</sup> CTL are induced by this vaccine regimen. A detailed comparative analysis of the functional properties of these two types of *env*-specific CTL has led to a number of conclusions that have implications for HIV-1 vaccine design and for AIDS pathogenesis.

One particularly striking finding was that the *env*-specific CTL induced by the *vac-env*/gp160 regimen are extremely effective in lysing target cells expressing HIV-1 *env* antigen. CD8<sup>+</sup> CTL cloned from a *vac-env*/gp160 recipient functioned impressively in vitro, lysing *env*-expressing target cells at exceedingly low E/T ratios (0.03:1). This indicates that individual vaccine-induced CTL can readily lyse multiple target cells during the assay period. CD8<sup>+</sup> CTL were several-fold more efficient on a per cell basis than CD4<sup>+</sup> CTL. Even more remarkable was the fact that the culture from which these clones were derived demonstrated *env*-specific cytolytic activity at E/T ratios as low as 0.1:1 (Fig. 2 A). These assays were performed after a single in vitro stimulation. Thus, the extraordinary lytic activity observed in this culture did not result from selection by repeated in vitro stimulation. Rather, it reflects both the high lytic activity of individual *env*-specific CTL clones induced by this vaccine regimen and the high frequency of such clones in the culture. Because the cloning step was carried out using a nonspecific mitogen that effectively stimulates all T cells regardless of their phenotype or specificity (45), the properties of resulting clones reflect the composition of the culture, that is of cells responding to the initial in vitro stimulation with *env*-transfected stimulating cells. Of the clones isolated, a remarkable 36% were positive for *env*-specific cytolytic activity. Most of these were CD8<sup>+</sup>. As is discussed below, it is likely that the high frequency of *env*-specific CTL in this culture reflects not only the high frequency of memory *env*-specific CTL among PBMC from vaccinees, but also a rapid clonal expansion of these *env*-specific CTL in response to stimulating cells expressing *env* antigen. In any event, although only a small number of volunteers have been immunized with this vaccine regimen to date, the data presented here demonstrate that this approach can lead to the generation of extremely vigorous CD8<sup>+</sup> CTL responses against cells expressing HIV-1 *env* antigen in at least some vaccinees.

Another unique feature of the present study is that a combined live virus/purified protein vaccine regimen was used. Since vaccination of humans with purified recombinant forms

of the HIV-1 envelope protein has been previously shown to induce a T cell response that is exclusively from the CD4<sup>+</sup> subset (18, 60), it is likely that the induction of CD8<sup>+</sup> CTL in these volunteers resulted from the initial *vac-env* immunization. CD4<sup>+</sup> CTL specific for the *env* protein can be induced by immunization with either *vac-env* (P. E. Stanhope and R. F. Siliciano, unpublished observations) or with purified gp160 (18). In addition to lysing infected cells, many *env*-specific CD4<sup>+</sup> T cells secrete IL-2 and may thereby facilitate the clonal expansion of CD8<sup>+</sup> CTL specific for the same antigen. In some systems, the generation of an effective CD8<sup>+</sup> CTL response upon viral challenge requires CD4<sup>+</sup> T cell help (61–64). Because the HIV-1 envelope protein synthesized in infected cells is processed for association with both class I and class II MHC molecules (18, 44), and because all of the cells that are productively infected in vivo express both class I and class II MHC molecules, infected cells should be capable of simultaneously stimulating both CD8<sup>+</sup> and CD4<sup>+</sup> T cells. Since, as shown here, the *vac-env*/gp160 regimen induced both CD8<sup>+</sup> and CD4<sup>+</sup> *env*-specific T cell clones, it is possible that the extremely intense CD8<sup>+</sup> CTL response observed after in vitro stimulation with *env*-expressing stimulator cells was due in part to the ability of *env*-specific CD4<sup>+</sup> T cells to promote the clonal expansion of *env*-specific CD8<sup>+</sup> CTL. If the same mechanism operates in vivo in vaccinated individuals who are exposed to HIV-1, the resulting CD8<sup>+</sup> CTL response may be of sufficient intensity to cause the rapid elimination of productively infected cells.

In addition to testing the lytic activity of vaccine-induced CTL clones against the standard *vac-env*-infected targets, the interaction of *env*-specific CD8<sup>+</sup> CTL clones with HIV-1-infected autologous CD4<sup>+</sup> T cells was evaluated directly using an in vitro model of HIV-1. The target cells in this model were autologous, nontransformed CD4<sup>+</sup> T lymphoblasts. Previous in vitro studies have demonstrated that CD8<sup>+</sup> CTL isolated from HIV-1-infected individuals lysed HIV-1-infected macrophages and CD4<sup>+</sup> T cells (3, 65), and that CD4<sup>+</sup> CTL induced by immunization of seronegative humans with gp160 could lyse autologous HIV-1-infected CD4<sup>+</sup> lymphoblasts (18). The data presented here indicate that vaccine-induced CD8<sup>+</sup> CTL efficiently lyse autologous HIV-1-infected cells.

Data presented here also establish that vaccine-induced CD8<sup>+</sup> CTL do not mediate the gp120-dependent lysis of noninfected CD4<sup>+</sup> T cells. As shown here and in previous studies (18, 37), free gp120 can bind to CD4 on activated human CD4<sup>+</sup> T cells and then be internalized and processed for presentation in association with MHC class II molecules, leading to lysis of the cells by gp120-specific CD4<sup>+</sup> CTL. However, the present study demonstrates that the same targets are not lysed by gp120-specific CD8<sup>+</sup> CTL from the same donor. This result is consistent with the notion that, with rare exceptions (66, 67), the processing pathway for exogenous antigen does not allow for association with MHC class I molecules (14–17). These results were confirmed using an in vitro model of HIV-1 infection of activated autologous CD4<sup>+</sup> T cells. In cultures of acutely infected CD4<sup>+</sup> lym-



phoblasts, it was shown that although only a minority of the cells become productively infected, all of the cells stain with an anti-gp120 antibody. This is the result of gp120 release from infected cells and/or HIV-1 virions, and subsequent CD4-dependent uptake by other cells (37). Most importantly, it was demonstrated that vaccine-induced gp120-specific CD4<sup>+</sup> CTL lysed a fraction of target cells that was substantially greater than the fraction of productively infected cells, while CD8<sup>+</sup> CTL lysed only the productively infected cells. In both cases, the lysis was antigen specific since no lysis was observed for targets that had not been initially exposed to HIV-1. The observed lysis of noninfected cells by gp120-specific CD4<sup>+</sup> CTL does not represent the classic form of "innocent bystander killing," which involves the release by antigen-activated CTL of a stable diffusible mediator that can lyse other cells in a nonspecific fashion. In fact, vaccine-induced CD4<sup>+</sup> CTL do not mediate this type of innocent bystander killing and instead lyse target cells in a highly specific, contact-dependent fashion through a mechanism that is indistinguishable from that used by the classic CD8<sup>+</sup> CTL (E. P. Miskovsky, P. E. Stanhope, and R. F. Siliciano, manuscript in preparation). The lysis of noninfected cells observed in this system actually represents the gp120-specific lysis of cells expressing processed gp120 determinants in association with class II MHC molecules. Thus, gp120-specific CD4<sup>+</sup> CTL are unable to distinguish between infected cells and noninfected CD4<sup>+</sup> T cells that have simply taken up exogenous gp120. Because these observations were made in the context of an actual *in vitro* HIV-1 infection, they heighten concern over the possible role of gp120-specific CD4<sup>+</sup> CTL in the process of CD4<sup>+</sup> T cell depletion in infected individuals. It is also important to note, however, that these data establish that gp120-specific CD8<sup>+</sup> CTL do not mediate this potentially deleterious reaction.

This study also addresses a major problem in HIV-1 vaccine development: that of HIV-1 genomic heterogeneity. The

ability of a vaccine to induce CTL crossreactive with a wide range of HIV-1 isolates is of obvious importance. Even if a vaccine does not prevent initial infection, the presence of vaccine-induced CTL directed at conserved epitopes may alter the course of infection in a favorable way. Nowak et al. (68) recently proposed that there is an antigen diversity threshold below which the virus load can be controlled by the immune system but above which the virus load induces the imminent destruction of the immune system. A vaccine that sensitizes the immune system to a diverse set of isolates in effect raises the viral antigenic diversity threshold below which the virus population can be regulated. This is important in light of recent evidence for CTL escape variants in natural infection (69). Thus, it is encouraging to note that CD8<sup>+</sup> CTL clones active against divergent isolates of HIV-1 were readily detected among the small set of vaccine-induced clones analyzed here. Crossreactive *env*-specific CD8<sup>+</sup> CTL have also been detected in HIV-1-infected individuals (4, 6, 70). In these studies, screening was carried out using *vac-env* vectors developed from the LAI isolate. Therefore, only crossreactive CTL were detected, and it is unclear what fraction of the total *env*-specific CD8<sup>+</sup> CTL response in natural infection consists of clones recognizing conserved epitopes. It is therefore particularly promising that highly crossreactive CTL were readily detected in the vaccine setting without special screening. Of course, the crossreactivity of individual vaccine-induced CTL depends upon the epitope recognized and will therefore vary with the MHC genotype of the vaccinee.

Although the recruitment of vaccinia-naive, seronegative volunteers represents a considerable challenge, the encouraging results of the present study and of studies of antibody and T cell proliferative responses in *vac-env*/gp160 recipients (B. Graham et al., manuscript submitted for publication; P. Greenberg et al., manuscript submitted for publication) suggest that further trials of this type of vaccine regimen in large numbers of human volunteers should be carried out.

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