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Enhancement of human immunodeficiency virus (HIV)-specific CD4$^+$ and CD8$^+$ cytotoxic T-lymphocyte activities in HIV-infected asymptomatic patients given recombinant gp160 vaccine

(AIDS/vaccination)

SMRITI K. KUNDU*, DAVID KATZENSTEIN*, LINCOLN E. MOSES†, AND THOMAS C. MERIGAN*‡

*AIDS Clinical Trials Unit, Center for AIDS Research, and †Division of Biostatistics, Stanford University, Stanford, CA 94305

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ABSTRACT Twenty-six human immunodeficiency virus (HIV)-infected asymptomatic patients with CD4$^+$ lymphocytes >400 per mm^3 were randomly allocated to a range of doses of recombinant gp160 or a control (recombinant hepatitis B vaccine) on a double-blind basis. Each patient received an injection at 0, 4, 12, 24, 36, and 48 weeks. Treatment assignments were decoded when all patients reached 28 weeks of the study period. HIV-1-specific CD4$^+$ and CD8$^+$ cytotoxic T lymphocyte (CTL) activities were assessed *in vitro* before vaccination and 2 weeks after each injection. There were significant increases in major histocompatibility complex-restricted HIV-1 Env-specific CD4$^+$ and CD8$^+$ CTL activities in 18 of 21 gp160 vaccinees. No control-injected patients showed a significant change. Neither gp160 nor control recipients showed significant changes in HIV-1 Gag- and Pol-specific CTL activities. HIV-1 Env-specific CD4$^+$ and CD8$^+$ CTL precursor frequencies were also measured in three vaccinees before and at 24 weeks after vaccine was started. CTL precursor frequencies also increased in both CD4$^+$ and CD8$^+$ populations. This study shows that this gp160 vaccine is immunogenic in enhancing HIV-1 Env-specific cytotoxic T-cell-mediated immunity in HIV-seropositive individuals.

Immunization of human immunodeficiency virus (HIV)-infected individuals with a recombinant gp160 vaccine is an experimental approach to active immunotherapy of an established retroviral infection. Redfield and his colleagues (1) have demonstrated that injection of an HIV-1 envelope gp160 vaccine can elicit additional antibody and lymphocyte proliferation responses against gp160 in HIV-infected individuals. However, successful clinical trials of HIV vaccine therapy have to overcome the issue of a significant diversity of the envelope glycoprotein of the virus within various patients (2). Another problem is the interpretation of these trials because of the variable and chronic clinical course of HIV infection. Finally, the specific immune responses that limit the progression of HIV infection have not been clearly defined.

The generation of major histocompatibility complex (MHC)-restricted cytotoxic T lymphocyte (CTL) activity is an important cell-mediated immune mechanism that plays an important role in the response to viral infections and recovery from virus-induced disease. HIV infection has been shown to elicit CD4$^+$ and CD8$^+$ CTL that recognize *env, gag, pol, tat,* and *nef* gene products (3–8). This HIV-specific CTL activity has also been shown to decline during the progression from asymptomatic infection to AIDS (9, 10).

In this study, HIV-seropositive individuals were immunized with recombinant gp160 from HIV-1. Effects of this vaccine on HIV-specific CTL responses against HIV *env*, *gag*, and *pol* gene-expressing autologous targets and CTL precursor frequency were determined.

MATERIALS AND METHODS

Study Population. The 26 HIV-infected, asymptomatic individuals included in this study represent the Stanford cohort within AIDS Clinical Trials Group trial 137, and the combined results on the shared tests with New York University cohort will be published later. These patients had CD4$^+$ T lymphocytes of >400 per mm^3. No patient had received any antiretroviral or immunomodulatory drug before or during the trial.

Vaccines. HIV-1 vaccine is a subunit gp160 vaccine, derived from HIV-1 lymphoadenopathy-associated virus isolate (VaxSyn, MicroGeneSys, Meriden, CT). The gp160 is a baculovirus-expressed recombinant protein produced in the cells of lepidopteran insects and then purified; the protein was mixed with the adjuvant aluminum phosphate. Doses ranging from 20 to 1280 μg of gp160 were used. Control vaccine was recombinant hepatitis B surface antigen (Recombivax HB; Merck Sharp & Dohme). Groups of 5, 10, 5, 1, and 5 patients received 1280, 320, 80, and 20 μg of gp160 vaccine and hepatitis B vaccine, respectively. Each patient received vaccine at 0, 4, 12, 24, 36, and 48 weeks as an i.m. injection.

MHC-Restricted HIV-1 Env-, Gag-, and Pol-Specific CTL Activities. HIV-1 Env-, Gag-, and Pol-specific CTL activities were measured before and 2 weeks after each injection by ^{51}Cr-release assay (8). Targets were autologous Epstein–Barr virus-transformed B-lymphoblastoid cell lines incubated for 16 hr with vaccinia recombinants (vSC8, vPE16, VV:gag, or vCF21) expressing LacZ, HIV-1 Env, HIV-1 Gag, or HIV-1 Pol antigens, respectively (AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases); 10 multiplicity of infection units was used for each recombinant. Eighty-five percent to 90% of target cells expressed these antigens and were used for the CTL assay. These target cells were labeled with ^{51}Cr (specific activity, 250–500 μCi; 1 Ci = 37 GBq). Spontaneous release of ^{51}Cr was 10–15% of maximum release.

Peripheral blood mononuclear cells were obtained from fresh peripheral blood by Ficoll/Hypaque centrifugation (11). T cells were separated from peripheral blood mononuclear cells by passage through a nylon wool column (12). Then CD4$^+$ and CD8$^+$ T cells were obtained by indirect panning (13) and were afterward treated with anti-CD4 or anti-CD8 monoclonal antibody and guinea pig complement

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Abbreviations: HIV, human immunodeficiency virus; MHC, major histocompatibility complex; CTL, cytotoxic T lymphocyte(s).

‡To whom reprint requests should be addressed at: Room S-156, Center for AIDS Research, Stanford University, Stanford, CA 94305.

(8). Purity of the cells was determined by indirect immunofluorescence assay (8). The cells used as effectors in the experiments were >90% pure; the remaining 10% of the cells was a mixture of B cells, natural killer cells, and monocytes (≈2% each). Effector-to-target ratios were 100:1, 50:1, 25:1, and 12.5:1. Target/effector mixtures were incubated for 6 hr and then harvested; radioactivity was counted in the γ counter. HIV-1 Env-, Gag-, or Pol-specific cytotoxicities were calculated as described earlier (8). Briefly, vac–*lac*-specific cytotoxicity (from recombinant vSC8) was subtracted from vac–*env*-, vac–*gag*-, or vac–*pol*-specific cytotoxicities to determine HIV-1 Env-, Gag-, or Pol-specific cytotoxicities, respectively. Vac–*lac*-specific cytotoxicity was ≈10%. Ten percent HIV antigen-specific lysis was used as the basis for a significant response because 10 HIV seronegative people showed nonspecific lysis of ≈10% with vac–*lac*-, vac–*env*-, vac–*gag*-, vac–*pol*-infected autologous Epstein–Barr virus-transformed B-lymphoblastoid cells. We have further characterized the role of MHC antigens in these cytotoxicities, as described (8).

HIV-1 Env-Specific CTL Precursor. HIV-1 Env-specific CTL precursor frequencies were measured in three vaccinees before and at 24 weeks of the study period. These three individuals were chosen randomly, and they were positive for Env-specific CTL activity in bulk assay. CTL precursor frequencies were measured by limiting-dilution analysis (14).

Limiting-dilution cultures were prepared by adding 1×10^4 heterologous irradiated peripheral blood mononuclear cells as a feeder layer in round-bottomed 96-well plates. $CD4^+$ and $CD8^+$ T lymphocytes were resuspended in RPMI 1640 medium containing gp160 at 1 μg/ml, 10% fetal calf serum, and 100 units of recombinant human interleukin 2 (Cetus) and added to the wells at concentrations of 10^5, 5×10^4, 10^4, 5×10^3, 10^3, and 0 cells per well. Twenty-four replicate wells were incubated at each cell concentration. Limiting-dilution cultures were incubated for 10–12 days with addition of medium containing gp160 and interleukin 2 every 3–4 days. The phenotypes of the T lymphocytes derived in limiting-dilution cultures were assessed by immunofluorescence staining with anti-CD4, anti-CD8, and anti-CD16 monoclonal antibodies and flow cytometry analysis at the time the cells were used as effectors. On day 10–12, each well was split and assayed for cytotoxicity on ^{51}Cr-labeled vac–*lac* and vac–*env* target cells in parallel, in a 6-hr ^{51}Cr-release assay. Individual wells were scored positive when ^{51}Cr release exceeded the mean of negative control wells by 3.0 SDs. This test was also done with vac–*gag* and vac–*pol* target cells to determine specificity of Env-specific CTL precursors. CTL precursor frequencies were estimated by applying Poisson probability theory to the single-hit model; 95% confidence limit was determined for each estimate by using the statistical method of χ^2 minimization (15).

Statistical Analysis. We quantified the CTL responses as a single number by computing an indicator of maximum rise, which we called d, defined as the sum of the two highest values seen minus twice the prevaccination value. Secondly, we calculated by least squares the slope (b) of the regression of the temporally ordered CTL changes upon time. A large value of either d or b was an indicator of elevated response after immunization. CTL changes were also calculated as fold changes. To ascertain whether CTL responses varied with dose, two approaches were used. (*i*) Each subject's slope b was characterized as high ($b > 0.2$) or low ($b < 0.2$) because $b = 0.2$ was the median value of all slopes, and the Mann–Whitney Wilcoxon rank–sum test for ordered contingency tables was applied (16). (*ii*) The individual values of d were arrayed against dose graphically to determine the dose–response.

RESULTS

MHC-Restricted HIV-1 Specific CTL Activities. Additional Env-specific $CD4^+$ and $CD8^+$ CTL activities appeared in 6 and 5 of 21 gp160 vaccinees, respectively (Table 1). Table 1 also shows individual patient's Env-specific $CD4^+$ and $CD8^+$ CTL activities and the week of peak response. The changes in Env-specific CTL activities were at least 2- to 3-fold greater than the prevaccine values in the responders (Fig. 1). Fig. 2 *A* and *B* shows the mean prevaccine and mean peak postvaccine levels of Env-specific cytotoxicities by $CD4^+$ and $CD8^+$ cells. Fig. 1 *A* and *B* shows the kinetics of the individual Env-specific cytotoxicities measured before (0) and 2 weeks after each injection in the 12 subjects who had received all six doses. Most patients showed a significant change by 14–26 weeks after three to four injections. Two vaccinees did not show any change in Env-specific $CD4^+$- and $CD8^+$-mediated cytotoxicities (Fig. 1 *A* and *B*). One vaccinee did not have >10% CTL activity before vaccination and did not develop additional responses. The other subject had Env-specific CTL activities (>10%) before vaccination but did not show any significant change after vaccination. The percent cytotoxicities shown by the dotted line were of subjects receiving control; their CTL activities did not change significantly.

Linear-regression analysis of Env-specific $CD4^+$ and $CD8^+$ CTL activities showed that patients receiving gp160 vaccine had higher slope values (>0.2) than the patients receiving control. Changes in $CD4^+$- and $CD8^+$-mediated Env-specific CTL activities were highly concordant. The evidence of a dose–response relation was found in the tendency for those receiving higher doses of gp160 also to be the patients with slopes exceeding $b = 0.2$. This relationship was assessed by the Mann–Whitney Wilcoxon rank–sum test; slope values showed a dose–response relationship (0.02

Table 1. HIV-1 Env-specific CTL responses to gp160 vaccine

		Env-specific CTL			
Vaccine	Patient	CD8	Week	CD4	Week
gp160					
1280 μg	1	+*	14	+*	14
	2	++	26	+	38
	3	++	14	+	14
	4	+*	26	+*	26
	5	+	14	+*	14
320 μg	6	++*	14	+	14
	7	+	26	+	38
	8	+	38	−	
	9	+	14	+	14
	10	+*	14	+*	26
	11	+	38	+	38
	12	+	2	+	14
	13	−		−	
	14	−		−	
	15	+	14	+	14
80 μg	16	+	6	++*	14
	17	+*	26	+*	26
	18	+	14	+	14
	19	+	14	+	38
	20	+	26	+	6
20 μg	21	+	14	+	14
Hepatitis B	22	−		+	14
	23	+	2	−	
	24	−		−	
	25	−		−	
	26	ND		ND	

Peak responses were shown at the indicated weeks. ++, >50; +, 11–50; −, <10.
*Env-specific CTL response developed after vaccination.

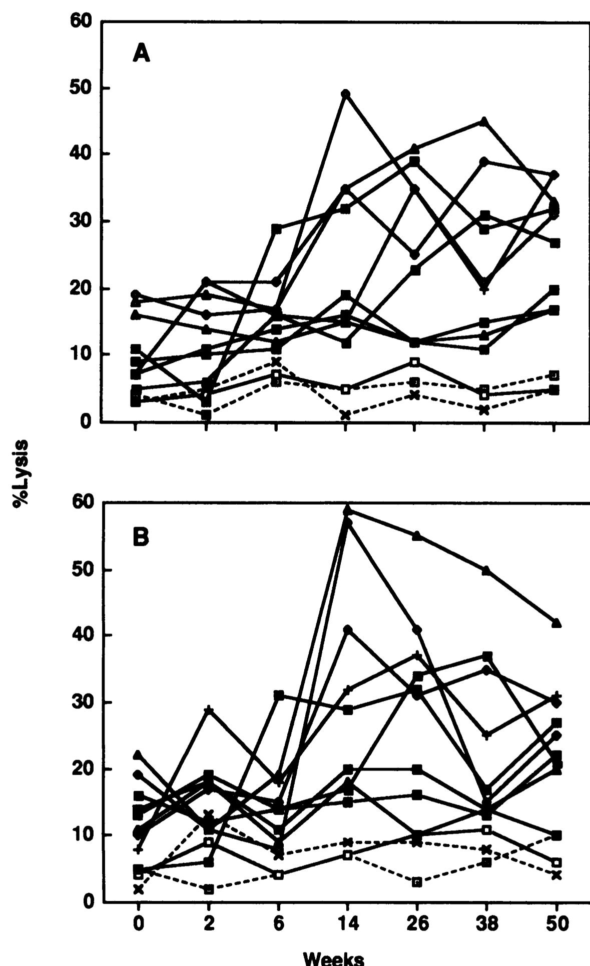

FIG. 1. Kinetics of HIV-1 Env-specific $CD4^+$ (*A*) and $CD8^+$ (*B*) CTL responses on vaccination. - - -, Responses of hepatitis B vaccine recipients; ——, responses of gp160 recipients. Responses were measured before (0) and 2 weeks after each injection.

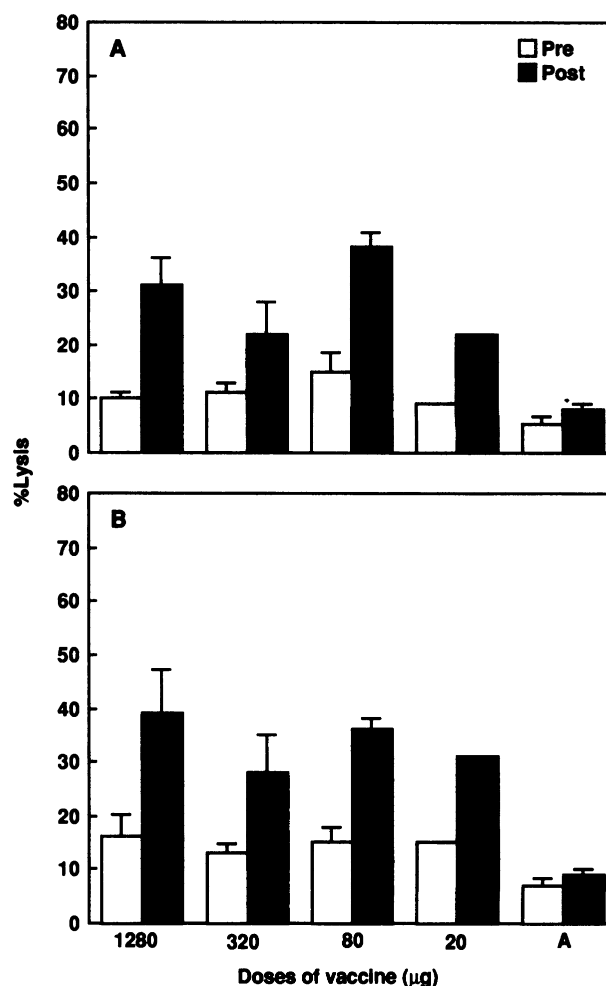

FIG. 2. Effect of recombinant, subunit gp160 vaccine on HIV-1 Env-specific $CD4^+$ (*A*), $CD8^+$ (*B*) CTL responses in HIV-infected asymptomatic patients. Number of patients was 5, 10, 5, 1, and 4 at 1280, 320, 80, and 20 µg of gp160 and hepatitis B vaccine (A bars), respectively. Env-specific CTL activity was obtained by subtracting vac–*lac* $CD4^+$, $CD8^+$ CTL from vac–*env* $CD4^+$, $CD8^+$ CTL activities. Targets were autologous Epstein–Barr-transformed B-lymphoblastoid cell lines infected with recombinant vSC8 (vac–*lac*) or recombinant vPE16 (vac–*env*). Mean (+SEM) of prevaccine (□) and highest postvaccine (■) (see Table 1) CTL activities at each dose of gp160 and hepatitis B vaccine were shown at target/effector ratio of 1:50.

$< P < 0.05$). But no dose–response relationship was observed with *d* values. There was no significant change in the HIV-specific CTL activities in the control group. Therefore, the increase in Env-specific CTL activities was produced by gp160 vaccine.

We have also tested Gag- and Pol-specific $CD4^+$ and $CD8^+$ CTL activities. Fig. 3 shows the mean prevaccine and mean peak postvaccine values of HIV-1 Gag- and Pol-specific CTL activities mediated by $CD4^+$ and $CD8^+$ cells. There was a trend toward increases in these CTL activities, which were significantly less than the Env-specific CTL activities, and these increments appeared after Env-specific CTL had reached a peak value in the gp160 vaccinee. The changes in Gag- and Pol-specific CTL activities were less than twice the baseline values. Slope values of these CTL activities with time were significantly lower (<0.05) than that of Env-specific CTL activities in gp160 recipients. Control recipients did not show changes in Gag- and Pol-specific CTL activities. The HIV-specific $CD4^+$ and $CD8^+$ CTL activities were MHC class II- and class I-restricted, respectively (8).

HIV-1 Env-Specific CTL Precursors. HIV-1 Env-specific CTL precursors were present in both $CD4^+$ and $CD8^+$ populations (Table 2). Gag- and Pol-specific cytotoxicity was not observed (results not shown). There was a significant increase in both $CD4^+$ and $CD8^+$ CTL precursors after gp160 vaccination (Mann–Whitney Wilcoxon rank–sum test, $0.02 < P < 0.05$). The cytotoxicity was MHC-restricted (results not shown). The stability of expression of $CD4^+$ or $CD8^+$ phenotype on >99% of the proliferating cells was documented by flow cytometry analysis at the end of the incubation period for each limiting-dilution culture.

DISCUSSION

We report here the enhancement of MHC-restricted HIV-1 envelope-specific $CD4^+$ and $CD8^+$ CTL responses induced by postinfection immunotherapy with a recombinant gp160 vaccine. This vaccine has been studied in seronegative individuals (17–20) as well as in a previous study as an immunotherapeutic agent in HIV-seropositive individuals (1). In both seronegative and seropositive vaccine recipients, increases in lymphocyte proliferative responses to gp160 and anti-envelope antibodies have been seen (1, 17–19). Orentas *et al.* (20) had shown an increase in $CD4^+$ Env-specific CTL activities in HIV-seronegative individuals. In this study, $CD4^+$ and $CD8^+$ CTL effector cell responses were documented in asymptomatic, HIV-infected individuals. Enhancement of these responses was seen in 18 of 21 vaccine recipients, whereas control recipients showed no change

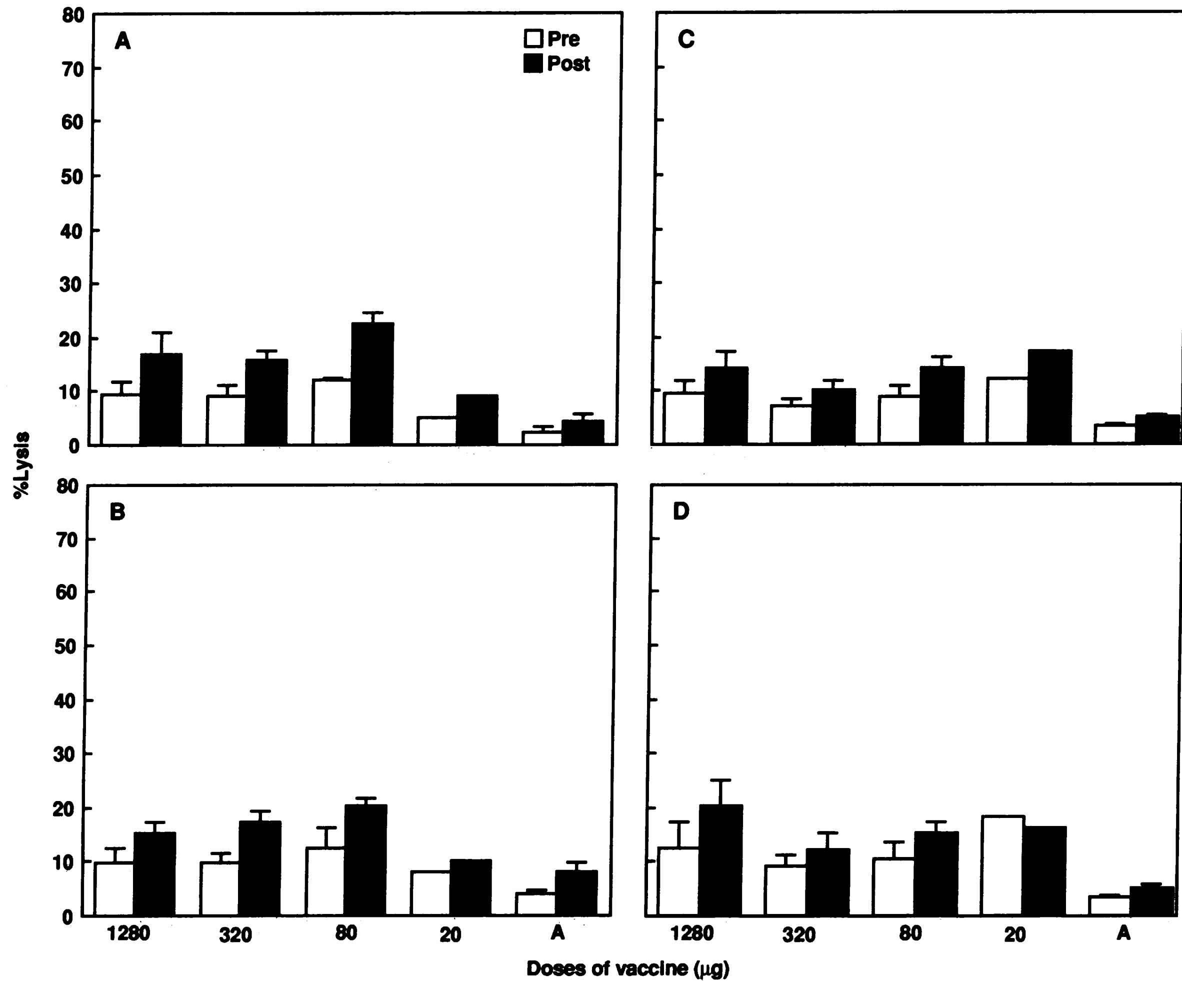

FIG. 3. HIV-1 Gag- and Pol-specific $CD4^+$ (*A* and *C*) and $CD8^+$ (*B* and *D*) CTL activities, respectively, in HIV-infected asymptomatic patients before and after vaccination. Gag- and Pol-specific CTL activities were detected as described for Fig. 1. Targets were autologous Epstein–Barr virus-transformed B-lymphoblastoid cell lines infected with recombinant vSC8 (vac–*lac*), VV:gag (vac–*gag*), or vCF21 (vac–*pol*). Mean (+SEM) prevaccine (□) and highest postvaccine (■) CTL activities at each dose of gp160 and hepatitis B vaccine were shown at target/effector ratio of 1:50. Numbers of patients were same as for Fig. 2.

(Table 1). The frequency of Env-specific cytotoxic precursor cells was also studied in detail in three gp160 vaccine recipients. The limiting-dilution analysis showed that the number of circulating CTL precursors was similar in $CD4^+$ and $CD8^+$ populations and that both CTL precursor frequencies increased after vaccination (Table 2).

The observed increase in Env-specific CTL activities after immunization could occur by several mechanisms. (*i*) Both endogenous and exogenous antigens can be processed and presented to both $CD4^+$ and $CD8^+$ CTLs (21). (*ii*) We have also observed that the production of cytokines, such as interleukin 2 and interferon-γ, by peripheral blood mononuclear cells on gp160 stimulation increases after immunization (unpublished work). In the Stanford cohort, the production of cytokines was measured 2 weeks after each injection—that is, at the same time of CTL assays. The enhanced cytokine productions were inter-correlated with increased CTL activities (analysis of covariance, $r = 0.6$) (data not shown). These cytokines may, therefore, play a role in increased Env-specific CTL activities. Increases in HIV Gag- and Pol-specific CTL activities after vaccination with gp160 could result from lysis of HIV-infected cells by Env-specific CTL,

Table 2. HIV-1 Env-specific CTL precursor frequencies before and after gp160 vaccination

Patient	CTL precursor frequency*			
	CD4		CD8	
	Prevaccine	Postvaccine	Prevaccine	Postvaccine
1	1:23,000 (1:17,000–1:32,000)	1:4900 (1:3910–1:6000)	1:17,000 (1:10,000–1:26,000)	1:2300 (1:1500–1:2900)
2	1:13,000 (1:9000–1:19,000)	1:4000 (1:3000–1:5900)	1:24,000 (1:17,000–1:35,000)	1:7000 (1:4500–1:8500)
3	1:35,000 (1:19,000–1:45,000)	1:7000 (1:4500–1:8500)	1:28,000 (1:19,000–1:37,700)	1:4000 (1:3000–1:5900)

Numbers in parentheses indicate the range at the 95% confidence limit.

*Mann–Whitney Wilcoxon rank-sum test for statistical significance between pre- and postvaccine changes, $0.02 < P < 0.05$.

leading to the stimulation of Gag- and Pol-specific CTL activities. Alternatively, a general enhancement of T_{helper}-cell activity could lead to the increase in Gag and Pol CTL function.

The critical elements of the immune system that may protect against HIV infection or disease progression have not been well-defined. Anti-HIV antibodies appear to increase with duration of infection, and high titers of neutralizing antibodies are seen in patients with AIDS (22, 23). In contrast, cell-mediated immune responses—e.g., CTL activity—decrease with disease progression (9, 10), and there is progressive loss of the response to recall antigens, followed by the loss of alloreactivity (24). Although these changes have not been well-studied in terms of specific CTL versus specific T_{helper}-cell functions, clearly the decline in virus-specific CTL parallels the loss of $CD4^+$ helper function in the progression of HIV infection to AIDS. Some studies suggest that development of cellular or humoral immune responses in HIV infection could contribute to immunodeficiency through passive or active destruction of T cells (25–27). In other viral diseases, such as measles and respiratory syncytial virus, preexisting antibodies or immune responses induced by vaccination have been shown to exacerbate disease, rather than to provide protection (28, 29). Therefore, the possible induction of immunopathologic responses must be seriously considered in clinical trials of HIV vaccines.

However, both $CD4^+$ and $CD8^+$ CTLs probably play an important role in protective immunity against HIV and other opportunistic viral infections because HIV-specific CTL decreases with disease progression (9, 10). In this study, quantitative analysis of Env-specific cytotoxicity by limiting-dilution analysis showed that the number of circulating CTL precursors was similar in $CD4^+$ and $CD8^+$ T-cell populations, and these CTL precursor frequencies increased after vaccination (Table 2). Although it is difficult to extrapolate from *in vitro* studies of human viral immunity to *in vivo* conditions, reactivation of varicella zoster, cytomegalovirus, and herpes simplex virus is observed when $CD4^+$ T-cell numbers have declined, suggesting a role for $CD4^+$ CTL in maintaining an asymptomatic stage of HIV infection (14). Ho *et al.*[§] have also suggested the beneficial effects of autologous $CD8^+$ CTLs in AIDS patients in respect to disease progression. Therefore, the increase in number of CTL precursors and CTL activities after vaccination seen in this study is encouraging and could be much more cost-efficient in producing disease control than the cell-transfer method would be.

Generally, natural infection elicits an appropriate and protective immune response against the infecting microorganism. However, under certain circumstances this immune response can be modified and, perhaps, improved by postinfection vaccination. Most of our study patients had low-level prevaccine Env-specific CTL activities. These patients also produced detectable amount of interferon γ and interleukin 2 on gp160 stimulation before vaccination. These activities increased significantly after vaccination (unpublished work). Thus, HIV infection induces cell-mediated immunity that can be augmented by vaccination, most likely through presentation of immunodominant epitopes of the envelope protein that have been identified for T_{helper} and $T_{cytotoxic}$ functions (30). Because cell-mediated immunity is an important factor in halting disease progression in other virus infections, a postinfection vaccine strategy could be a promising alternative therapy for AIDS. The important remaining issues include demonstration of the impact of this form of immunotherapy on HIV viral load *in vivo* and on the clinical progression of HIV disease.

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