

Activation of Virus Replication after Vaccination of HIV-1-infected Individuals

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Summary

Little is known about the factors that govern the level of HIV-1 replication in infected individuals. Recent studies (using potent antiviral drugs) of the kinetics of HIV-1 replication in vivo have demonstrated that steady-state levels of viremia are sustained by continuous rounds of de novo infection and the associated rapid turnover of CD4⁺ T lymphocytes. However, no information is available concerning the biologic variables that determine the size of the pool of T cells that are susceptible to virus infection or the amount of virus produced from infected cells. Furthermore, it is not known whether all CD4⁺ T lymphocytes are equally susceptible to HIV-1 infection at a given time or whether the infection is focused on cells of a particular state of activation or antigenic specificity. Although HIV-1 replication in culture is known to be greatly facilitated by T cell activation, the ability of specific antigenic stimulation to augment HIV-1 replication in vivo has not been studied. We sought to determine whether vaccination of HIV-1-infected adults leads to activation of virus replication and the targeting of vaccine antigen-responsive T cells for virus infection and destruction. Should T cell activation resulting from exposure to environmental antigens prove to be an important determinant of the steady-state levels of HIV-1 replication in vivo and lead to the preferential loss of specific populations of CD4⁺ T lymphocytes, it would have significant implications for our understanding of and therapeutic strategies for HIV-1 disease. To begin to address these issues, HIV-1-infected individuals and uninfected controls were studied by measurement of immune responses to influenza antigens and quantitation of virion-associated plasma HIV-1 RNA levels at baseline and at intervals after immunization with the trivalent influenza vaccine. Influenza vaccination resulted in readily demonstrable but transient increases in plasma HIV-1 RNA levels, indicative of activation of viral replication, in HIV-1-infected individuals with preserved ability to immunologically respond to vaccine antigens. Activation of HIV-1 replication by vaccination was more often seen and of greater magnitude in individuals who displayed a T cell proliferative response to vaccine antigens at baseline and in those who mounted a significant serologic response after vaccination. The fold increase in viremia, as well as the rates of increase of HIV-1 in plasma after vaccination and rates of viral decline after peak viremia, were higher in individuals with higher CD4⁺ T cell counts. These data indicate that important aspects of the host-virus relationships underlying HIV-1 infection may be gleaned from the careful analysis of interventions that perturb, either positively or negatively, the steady-state equilibrium of virus replication in vivo. The potential adverse consequences of vaccine-induced activation of HIV-1 replication deserve consideration in formulating guidelines for immunization of HIV-1-infected individuals. Given the demonstrated ability of antigenic challenge to activate virus replication in infected individuals, the contribution of immune stimulation to T cell depletion and disease progression in HIV-1-infected individuals are important topics for future study.

Tissue culture studies of the HIV-1 replication cycle show that T cell activation and proliferation are required for efficient reverse transcription and nuclear import of viral preintegration complexes (1–3). Thereafter, transcription of the integrated provirus is activated by host cell transcription factors, most notably members of the nuclear factor κ B/rel family, which are induced by T cell activation (4, 5). In vivo, most of the HIV-1 viral burden and expression occur within lymphoid tissues at sites of the generation of the immune response (6). Virus is found in the germinal centers of lymph nodes and in the white pulps of the spleen (7–9). Recent studies of the dynamics of viral replication in vivo have elucidated high levels of virus replication underlying a steady state viremia in AIDS patients (10–12). However, little is known about what drives such substantial HIV-1 replication and determines the steady state levels of host–virus load.

We were interested in the possibility that antigenic activation might target specific antigen-responding CD4⁺ T cells for HIV-1 infection, virus production, and eventual destruction. Antigen-specific T cell depletion in HIV-1 infection could be driven by exposure to environmental antigens and might explain the preferential infection and early loss of memory T cells that have been reported during HIV-1 infection (13, 14). Demonstration of antigen-specific T cell depletion in HIV-1 infection would have important implications for our understanding of AIDS pathogenesis, as well as for potential strategies to ameliorate disease progression.

It is generally recommended that HIV-1-infected individuals be vaccinated against several important pathogens, including influenza viruses, *Streptococcus pneumoniae*, *Haemophilus influenzae*, and hepatitis B (15, 16). In addition, it is recommended that HIV-1-infected infants be vaccinated against diphtheria, tetanus, measles, mumps, rubella, polio, and pertussis. Although the efficacy of these vaccines in immunocompetent individuals has been established, the protective value of vaccination in the context of HIV-1 infection has not been demonstrated (17). It has been reported that many HIV-1-infected individuals do not make a significant antibody response to vaccine antigens, suggesting that routine vaccination of HIV-1-seropositive patients may be of little benefit. In most studies reported to date, the ability to respond to vaccination correlates with circulating CD4⁺ T cell levels, and decreases as CD4⁺ T cell counts decline and immunodeficiency becomes more severe (17, 18).

Previous studies, using less sensitive measures than are currently available, have variously suggested that immune stimulation may or may not result in increased HIV-1 expression in vivo (19–21). We report here the application of more sensitive virologic methods that demonstrated that influenza vaccination of HIV-1-infected individuals resulted in transient increases in plasma HIV-1 RNA levels. Increased viremia was more often seen and of greater mag-

nitude in individuals who displayed T cell proliferative responses to vaccine antigens at baseline and in those who mounted a significant serologic response after vaccination. Kinetic analyses of vaccine-induced changes in viremia were performed in an effort to elucidate the mechanism and consequences of antigen-induced activation of HIV-1 replication.

Materials and Methods

Study Population. After approval of the study protocol by the Internal Review Board of the Davies Medical Center, 32 subjects with HIV-1 infection and 10 control subjects confirmed to be HIV-1 antibody negative by ELISA were recruited from four private medical practices and a San Francisco Public Health Department Clinic. HIV-1-infected individuals were stratified by CD4⁺ T lymphocyte levels determined within 30 d of initiation of the study. Subject characteristics are detailed in Table 1. Informed consent was obtained. Blood samples were obtained on the day of the vaccination and on days 7, 14, 28, and at week 13 after vaccination for virologic and immunologic analyses. All samples were coded and analyzed by the laboratory in a blinded fashion.

Vaccination. All study participants received a standard dose (0.5 ml) of the 1993–1994 formulation of trivalent influenza vaccine (Fluzone split virus preparation; Connaught Laboratories,

Table 1. Characteristics of Study Participants

	CD4 lymphocyte group			Control subjects
	0–200	201–500	>500	
Number of subjects	10	11	11	10
Men	8	10	10	8
Women	2	1	1	2
Non-Caucasian	1	3	2	1
Age, yr (mean)	39	39	38	37
CD4 lymphocyte count				
Mean	57.6	343.8	679.8	N/A
Median	30.0	306.0	617.5	N/A
Range	0–186	260–495	529–1020	N/A
Previous flu vaccine				
Yes	8	8	8	3
No	2	1	2	7
Unknown	0	2	1	0
Antiretroviral therapy during study period				
Yes	6	9	4	N/A
No	4	2	7	N/A
AIDS by history of opportunistic infection				
Yes	5	0	0	N/A
No	5	11	11	N/A

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Swiftwater, PA) via intramuscular injection in the deltoid. The vaccine dose contained 15 µg hemagglutinin (HA)¹ of A/Texas/36/91 (H1N1), 15 µg HA of A/Beijing/32/92 (H3N2), and 15 µg HA of B/Panama/45/90. The HA of A/Beijing/32/92 replaced the HA of A/Beijing/353/89 (H3N2) that was included in the 1992–1993 formulation, whereas the A/Texas and B/Panama antigens were included in both the 1992–1993 and 1993–1994 formulations.

Sample Collection and Preparation. 10–30 ml of acid citrate dextran–anticoagulated blood was obtained from study participants and processed according to established procedures (22). For plasma isolation, blood was centrifuged for 20 min at 400 g, and then the plasma fraction was removed and recentrifuged for 5 min at 3,000 g to remove cellular debris. The clarified plasma samples were processed within 3 h of initial phlebotomy. PBMC were isolated by Ficoll–Paque (Pharmacia Biotech, Inc., Piscataway, NJ) density gradient centrifugation as described (22). Purified PBMC were viably cryopreserved in liquid nitrogen (22).

Quantitation of Plasma and Cellular HIV-1 RNA Levels. Levels of virion-associated HIV-1 RNA were measured using the HIV-1 branched DNA (bDNA) signal amplification method (Quantiplex HIV-1 RNA assay; Chiron Corp., Emeryville, CA) as described (23). Plasma samples yielding values of HIV-1 RNA levels below the bDNA cutoff (<10,000 copies HIV-1 RNA/ml of plasma) were reassayed by the HIV-1 quantitative competitive (QC) PCR method with the following modifications (24, 25): (a) HIV-1 RNA was isolated from virions pelleted from 1–3 ml of plasma using the Trizol (GIBCO BRL, Gaithersburg, MD) RNA extraction method. (b) For very low copy number samples, the competing RNA template series consisted of 0, 25, 50, 100, 500, 1,000, and 5,000 copies per reaction. (c) The thermal cycling conditions used were 94°C for 3 min (1 cycle); 93°C for 30 s, 55°C for 30 s, 72°C for 1 min (45 cycles); 72°C for 10 min (1 cycle); 4°C hold. (d) PCR products were radioactively labeled by the addition of [³²P]dCTP to the PCR reaction mix, followed by resolution of the PCR products by electrophoresis on 6% acrylamide gels and quantitation by phosphorimage analysis using a bioimager (FujixBas 1000 Bio-Imaging Analyzer; Fujix, Inc., Stamford, CT). With these modifications, the QC-PCR method has a sensitivity of ~100 copies of HIV-1 RNA/ml of plasma. Competition equivalence points were determined by interpolation on plots of the logarithm of the calculated ratio of signal for the wild-type target sequence–derived product (corrected for molar ratio) versus the logarithm of the copy number of added competitive template. For quantitation of HIV-1 mRNA present within PBMC, total cellular RNA was prepared from Ficoll–Hypaque–purified PBMC by the Trizol RNA extraction method. RNA, prepared from ~10⁵ PBMC, was reverse transcribed into cDNA and subjected to QC-PCR as described above.

Immunophenotyping. PBMC were obtained from acid citrate–anticoagulated blood samples by Ficoll–Hypaque separation. Aliquots of cells were stained with optimal concentrations of a panel of mAbs, as described (26). A minimum of 10,000 stained cells was analyzed on a FACScan[®] flow cytometer (Becton Dickinson & Co., Mountain View, CA) using two-color analysis and the Lysis II analysis software. Markers analyzed included CD3, CD4, CD8, CD45RA, CD45RO, and HLA-DR (mAb) reagents obtained from Becton Dickinson & Co.) to define various subpopulations of the CD4⁺ T cells (26). The results were expressed as a percentage of the lymphocytes stained with each mAb. Absolute

numbers of each population were calculated at the initiation of the study. CD45RA identifies a population of immature CD4⁺ T cells; CD45RO identifies a population of mature CD4⁺ T cells; and HLA-DR identifies a population of activated CD4⁺ T cells.

Cell-mediated Immune Responses. Cell-mediated immune responses to influenza and control antigens were assessed by the *in vitro* proliferation of PBMC to specific antigenic stimulation using a standard assay (26). Briefly, 10⁵ cryopreserved PBMC were cultured in U-bottom microtiter plates in RPMI 1640 medium supplemented with 25 mM HEPES, 10% human AB⁺ serum (Sigma Chemical Co., St. Louis, MO), 1% L-glutamine, 1% penicillin, and 1% streptomycin. A concentration of each antigen previously shown to yield optimal proliferative responses from human PBMC was added to appropriate wells, and the plates were incubated for 6 d at 37°C in a humidified atmosphere containing 5% CO₂. For the last 18 h of incubation, 1 µCi [³H]TdR was added to each culture. The cells were harvested onto glass fiber filters using a 96-well harvester and prepared for liquid scintillation counting in a counter (BetaPlate; Wallac, Gaithersburg, MD). Antigens used included the trivalent vaccine antigen (Connaught Laboratories) diluted 1:50, 1:100, and 1:500; tetanus toxoid (Connaught Laboratories) at a final concentration of 4 Lf/ml; and *Candida* antigen (Greer Labs, Lenoir, NC) at a final dilution of 10 µg/ml. The number of cells added to each well was adjusted to partially normalize the number of CD4⁺ T cells in each well. For subjects with CD4 counts >500, 10⁵ cells were added to each well. For subjects with CD4 counts between 200 and 500, 2 × 10⁵ cells were added, and 4 × 10⁵ cells were added for subjects with CD4 counts <200. The mean counts per minute from triplicate cultures were calculated. Results are described as a stimulation index equal to mean counts per minute of antigen-stimulated cultures/mean counts per minute of media control cultures. A stimulation index ≥2.5 was considered a positive proliferative response. This assay measures the proliferative response of mature CD45RO⁺CD4⁺ T memory cells. A positive response requires the previous *in vivo* stimulation of the T cell compartment with the specific antigen.

Antibody Response to Influenza Antigens. Plasma antibodies to influenza antigens were measured in a standard hemagglutinin inhibition assay using 0.5% chicken red blood cells as described (27). The assay was performed using monovalent influenza antigens from the 1993–1994 formulation kindly supplied by Wyeth–Ayerst Laboratories (Marietta, PA). Nonspecific inhibitors were removed from citrated plasma by treatment with Kaolin (28). The initial dilution of plasma was 1:10 with subsequent twofold dilutions. Titers were expressed as the reciprocal dilution giving complete inhibition of hemagglutinin. A significant increase in antibody titers was defined by a fourfold or greater increase in plasma antibody titer in samples obtained 4 wk after immunization.

Statistical Analyses. Proportional data were compared using Fisher's exact test. Correlations were determined using Spearman's rank correlation test. For analysis of the kinetics of change in plasma HIV-1 RNA levels, values of plasma viral RNA copy number were transformed to a natural log scale to normalize variance and to facilitate the use of linear analysis methods. The rate of viral population growth was estimated as the slope of the line drawn between the prevaccination viral copy number and the peak viral copy number after vaccination. The rate of viral population decline was estimated as the slope of the line drawn between the peak viral copy number and the first time point after peak viremia. These rates provide minimum estimates of growth and decline of the viral population because the real peak viremia may have been higher, but could not have been lower, than the

¹ Abbreviations used in this paper: bDNA, branched DNA; HA, hemagglutinin; HBV, hepatitis B virus; QC, quantitative competitive.

measured peak. Doubling time and half-life were calculated by dividing the natural log of 2 by the rates of viral population growth and decline, respectively. Linear regression was used to analyze associations between CD4⁺ T cell counts and the rates of viral population growth and decline. Analysis was performed using Statistical Analysis Software (SAS Institute, Inc., Cary, NC).

Results

Study Population and Design. 32 adult subjects with HIV-1 infection, equally distributed with respect to CD4⁺ T cell counts (0–1,020), and 10 seronegative control subjects were recruited from private and public medical clinics (see Table 1 and Materials and Methods). For data analysis, HIV-1-infected individuals were stratified by CD4⁺ T lymphocyte counts into three groups of 0–200, 201–500, and >500 CD4⁺ T lymphocytes/ μ l. All study subjects received a standard dose of the 1993–1994 formulation of the trivalent influenza vaccine via intramuscular injection, and blood samples were obtained before vaccination and on days 7, 14, 28, and at week 13 after vaccination for virologic and immunologic analyses. The mean age of the study participants was similar between the different groups, but HIV-1-infected participants were more likely to have been previously vaccinated against influenza. The majority of HIV-1-infected participants were receiving antiretroviral therapy (with the nucleoside analogues zidovudine, zalcitabine, didanosine, or stavudine monotherapy or various combinations thereof) before and during the study period. None of the study participants had evidence of active opportunistic infections at the time of study entry. There were no adverse clinical reactions to the influenza vaccine and there were no reports of an influenza-like illness.

Immunophenotyping. To determine whether there were any significant changes in the representation of lymphocyte subsets in the peripheral circulation after vaccination, we measured the cell surface expression of a number of lymphocyte markers. Considerable variation in the percentages of CD3⁺ (TCR⁺) T lymphocytes (range 39–90%), CD4⁺ T lymphocytes (range 1–48%), CD8⁺ T lymphocytes (range 22–67%), CD4⁺CD45RA⁺ (“naive”) T lymphocytes (range 12–97%), CD4⁺CD45RO⁺ (“memory”) lymphocytes (range 21–82%), CD4⁺HLA-DR⁺ (activated) T lymphocytes (range 2–31%), and CD4/CD8 ratios (range 0.02–2.18) were present in the peripheral blood of HIV-1⁺ subjects before vaccination. With the exception of the HLA-DR⁺ population of CD4⁺ T cells, the percentages of these T lymphocyte populations remained essentially constant for each individual HIV-1⁺ subject studied during the 4-wk observation period after vaccination. In the HIV-1⁺ individuals, the median percentage of HLA-DR⁺ T cells fell from 11 to 1% by 4 wk after vaccination ($P < 0.0001$). A decrease in the percentage of activated (HLA-DR⁺) CD4⁺ T lymphocytes was observed in 10 of 10 subjects who had elevated percentages at the beginning of the study. In contrast, the median percentage of HLA-DR⁺ CD4⁺ T cells in the seronegative controls remained constant at 1%. It is not possible to determine whether the ob-

served decrease in HLA-DR⁺ cells in HIV-infected individuals is due to loss or redistribution of the cells, but these changes are unlikely to be explained on the basis of an effect on influenza-specific T cells alone, which constitute only a tiny fraction of the circulating T cell population.

T Cell Proliferation. In vitro proliferation assays were performed to assess the integrity of T cell responses to the influenza antigens (14). In vitro proliferation of T cells is a measure of antigen-specific CD4⁺CD45RO⁺ T memory cells. Consistent with previous reports, the proportion of subjects responding to the influenza vaccine antigens and the magnitude of the proliferative response both increased with increasing CD4 counts (Fig. 1). At least some individuals in each study group responded to the influenza vaccine antigens in vitro: two of seven subjects (29%) with CD4 counts <200; 7 of 11 subjects (64%) with CD4 counts 200–500; and nine of nine subjects (100%) with CD4 counts >500. 4 wk after vaccination, diminished T cell proliferative responses were seen in most people, including seronegative control subjects, suggesting that the influenza-responsive cells are not present in the peripheral circulation at this time. This unexpected finding precluded us from determining whether vaccination may have actually resulted in a loss rather than an expansion of influenza-specific CD4⁺ T cells in HIV-1-infected individuals.

Antibody Responses to Vaccine Antigens. Antibody responses, an essential functional measure of the integrity of the CD4⁺ T cell-dependent immune response, were measured before and after vaccination. Poor antibody response to A/Texas/36/91 and B/Panama/45/90 were observed in all of the study groups, including the uninfected control subjects (Table 2). The A/Beijing/32/92 antigen was more immunogenic in all of the study groups. The proportion of subjects that made a significant increase in antibodies (defined as a fourfold or greater increase in titer) increased in proportion to the CD4 count. However, only the study group with CD4 counts <200 demonstrated a significantly

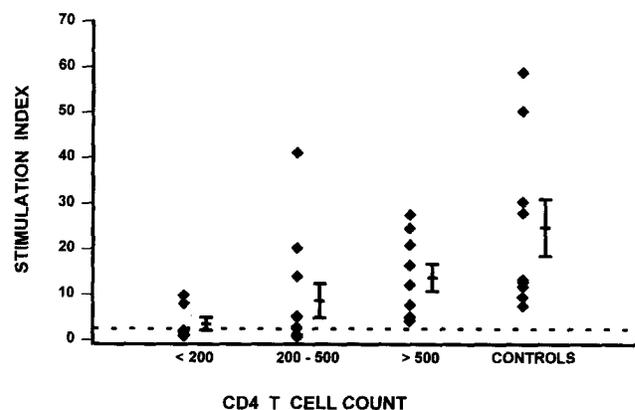


Figure 1. T cell proliferative response to influenza vaccine antigens, according to CD4 count. The proportion of subjects responding and the magnitude of the in vitro proliferative response of mature CD4⁺ T memory cells increases the CD4 count in HIV-seropositive subjects.

Table 2. Antibody Response to the 1993–1994 Influenza Vaccine in HIV-seropositive Subjects According to CD4 T Lymphocyte Count

Antigen	Group	n	Preimmunization titers*			Postimmunization titers		
			Mean	Range	Positive [‡]	Mean	Range	Responders [§]
					%			%
A/Texas/36/91	Control subjects	10	34.8	20–80	100	60.6	40–320	30
	All HIV ⁺	32	48.6	10–160	100	57.8	20–640	6
	CD4 <200	10	49.2	40–80	100	45.9	40–80	0
	CD4 200–500	12	50.4	20–320	100	53.4	40–80	0
	CD4 >500	10	45.9	10–160	100	80.0	20–640	20
A/Beijing/32/92	Control subjects	10	1.7	0–20	20	30.3	10–80	80
	All HIV ⁺	32	2.8	0–40	38	15.7	0–640	41
	CD4 <200	10	3.1	0–40	40	11.8	0–80	20
	CD4 200–500	12	3.3	0–20	42	15.0	0–160	42
	CD4 >500	10	2.1	0–20	30	22.0	0–640	60
B/Panama/45/90	Control subjects	10	45.9	20–160	100	105.6	40–320	30
	All HIV ⁺	32	68.7	20–320	100	110.7	40–1280	9
	CD4 <200	10	76.6	40–320	100	85.7	40–320	0
	CD4 200–500	12	59.9	20–160	100	95.1	40–320	8
	CD4 >500	10	74.6	40–320	100	171.5	80–1280	20

*Data presented as geometric range of antibody titers in hemagglutination inhibition units in samples obtained before and again 4 wk after immunization.

[‡]Percentage of subjects with detectable antibody in the preimmunization sample.

[§]Percentage of subjects with a four fold or greater increase in antibody titers after immunization.

^{||} $P < 0.05$ compared with control subjects by Fisher's exact test.

($P < 0.05$) lower proportion of responders (20%) compared with the control groups (80% responders).

All of the study subjects had detectable antibody to both the A/Texas/36/91 and B/Panama/45/90 antigens before vaccination with the 1993–1994 vaccine. Prevacination antibodies to the A/Beijing/32/92 antigen were present in 20% of the control and 38% of the seropositive subjects. These data most likely reflect the fact that the seropositive subjects had received routine influenza vaccination over the past several years, whereas the subjects in the control group were less likely to have been previously vaccinated against influenza.

Changes in Plasma Viremia. Levels of virion-associated HIV-1 RNA in the plasma are a good indicator of the level of ongoing virus replication in the body (10–12). We used the bDNA and QC-PCR methods to measure HIV-1 RNA in the plasma at weeks 0 (preimmunization), 1, 2, 4, and 13 relative to immunization (23–25). These two assays generate measures with a high degree of correlation (29 and our unpublished data). Taking into account the coefficient of variation within individual bDNA and QC-PCR assays, as well as the small biological variation in plasma HIV-1 RNA levels in clinically stable HIV-1-infected individuals, we considered a threefold change in the level of plasma HIV-1 RNA to be significant (23, 30). By this criterion, the majority (83%) of vaccinated individuals experi-

enced a significant increase in plasma HIV-1 RNA levels within 1–2 wk immunization and returned to their prevaccination levels within 4 wk after immunization (Table 3; $P = 0.0009$). The mean fold increase in HIV-1 RNA copy number was substantially greater in those individuals with higher CD4⁺ T cell counts (Fig. 2). At baseline, before immunization, the individual plasma HIV-1 RNA copy number measures covered a wide range (50–402,500 copies/ml). After immunization, the peak titers observed had a significantly more narrow distribution (range 5,800–1,600,000 copies/ml; Fig. 3; $P = 0.0009$). Among all HIV-1-infected participants, peak plasma HIV-1 RNA levels seen after vaccination ranged from 1- to 369-fold above baseline values (median 7.3). For individuals with <200 CD4⁺ T cells/ μ l,

Table 3. Percentage of Subjects with Greater than Threefold Increase in Plasma Viremia

Wk after immunization	CD4 <200	CD4 200–500	CD4 >500
1	30	36	60
2	78	67	90
4	50	17	40
13	33	27	0

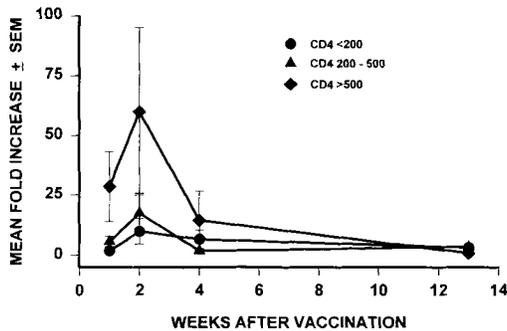


Figure 2. Tempo of plasma viremia by CD4 group. The mean fold increase in plasma HIV-1 RNA copy number at weeks 1, 2, 4, and 13 after influenza vaccination is shown. Bars indicate SEM.

peak postvaccination plasma HIV-1 RNA levels ranged from 1- to 52-fold above baseline values (median 4.0). Participants with 200–500 CD4⁺ T cells/ μ l showed peak postvaccination plasma HIV-1 RNA levels ranging from 1- to 89-fold above their baseline determinations (median 5.1). For those with >500 CD4⁺ T cells/ μ l, peak postvaccination elevations in plasma HIV-1 RNA levels ranged from 1.4- to 369-fold above baseline measurements (median 33). Patients on antiretroviral therapy were not noticeably different from those not in therapy with regard to increases in plasma viremia. In a few patients, plasma viremia did not return to baseline or showed a second increase during the study. None of these study participants were given a second vaccination during the study period, but these few subjects did have evidence of an intercurrent infection, such as the development of CMV retinitis or *Pneumocystis carinii* pneumonia, which may have caused the second wave of viremia.

To evaluate the impact of vaccination on rates of HIV-1 replication and clearance, we calculated the doubling times and half-lives of plasma virus from the rates of change of plasma HIV-1 RNA levels after vaccination. Rates of viral

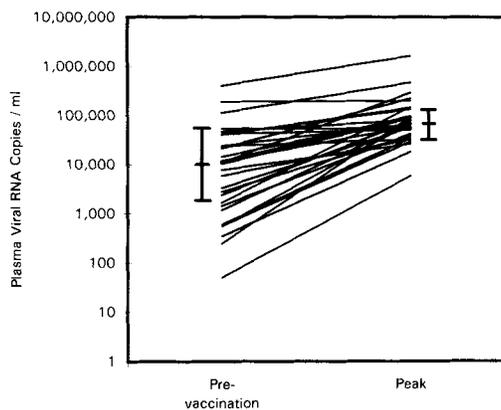


Figure 3. Baseline viremia before vaccination and the peak viremia occurring 7–21 d after vaccination. Each line represents one patient. Bars represent the median \pm the mean of the absolute differences from the median, which is a measure of variance. Median viremia was lower before vaccination (Wilcoxon two sample test, $P < 0.0001$) and was more variable (modified Levene median test, $P = 0.0009$ [54]). Statistical tests were performed after logarithmic transformation.

population growth were correlated with rates of viral population decline after peak viremia (Fig. 4 A; $P = 0.0002$). Subjects with higher baseline CD4⁺ T lymphocyte counts had higher rates of viral growth after vaccination (Fig. 4 B; $P = 0.0007$) and higher rates of viral decline after peak viremia (Fig. 4 C; $P = 0.01$). The fold increase in viremia was higher in patients with higher CD4⁺ T cell counts before immunization (Fig. 4 D; $P = 0.05$).

We also quantitated the levels of PBMC-associated HIV-1 RNA by QC-PCR at time points before and after immunization. There were no significant changes in PBMC HIV-1 RNA levels detected (data not shown), even in individuals with large increases in plasma viremia. The failure to detect elevations of HIV-1 RNA after vaccination suggests that the increase in plasma virus was originating from virus produced at other sites in the body, most likely the peripheral lymphoid organs, and that virus-producing cells do not survive to recirculate through the peripheral bloodstream.

Association between Immune Responses and Changes in Plasma Viremia. To evaluate whether there was any significant correlation between an immune response to influenza antigens and increases in plasma viremia, we performed statistical analysis of the data. A large increase (>10 \times) in plasma HIV RNA was observed in subjects who made an immune response to the vaccine antigens (Fig. 5). The correlation between the increase in plasma viremia and immune response was significant for both the presence of a T cell proliferative response to the vaccine antigens ($P = 0.01$) or the presence of a significant (fourfold or greater) increase in antibody titer ($P < 0.001$) to one or more of the vaccine antigens by Spearman rank order analysis. It should be noted that several individuals with low CD4⁺ T cell counts demonstrated both a measureable influenza-specific immune response and large increases in plasma viremia. Thus, it appears that vaccine stimulation of influenza-specific T cells in vivo leads to activation of HIV-1 replication.

Discussion

It is plausible that T cell depletion in HIV-1 involves specific antigen-driven loss of T cells as opposed to a more random process of T cell depletion. Pathogens that plague HIV-1-infected individuals early in the course of infection include those that are either prevalent in the environment (*Candida spp.*), or cause persistent infections (*Mycobacterium tuberculosis*, herpes simplex virus, varicella zoster virus) (31). The nature of the opportunistic infections seen in HIV-1-infected individuals and the timing with which these infections occur in the course of HIV-1 disease may be the result of the preferential depletion of CD4⁺ T cells that respond to commonly encountered antigens. Exogenous antigens (such as those that are prevalent in the environment) or endogenous antigens (such as those produced by latent infectious pathogens) might result in the frequent activation of host antigen-specific CD4⁺ T cells, thus increasing their susceptibility to HIV-1 infection and destruction. With time, the gradual attrition of the CD4⁺ T cell-protective response

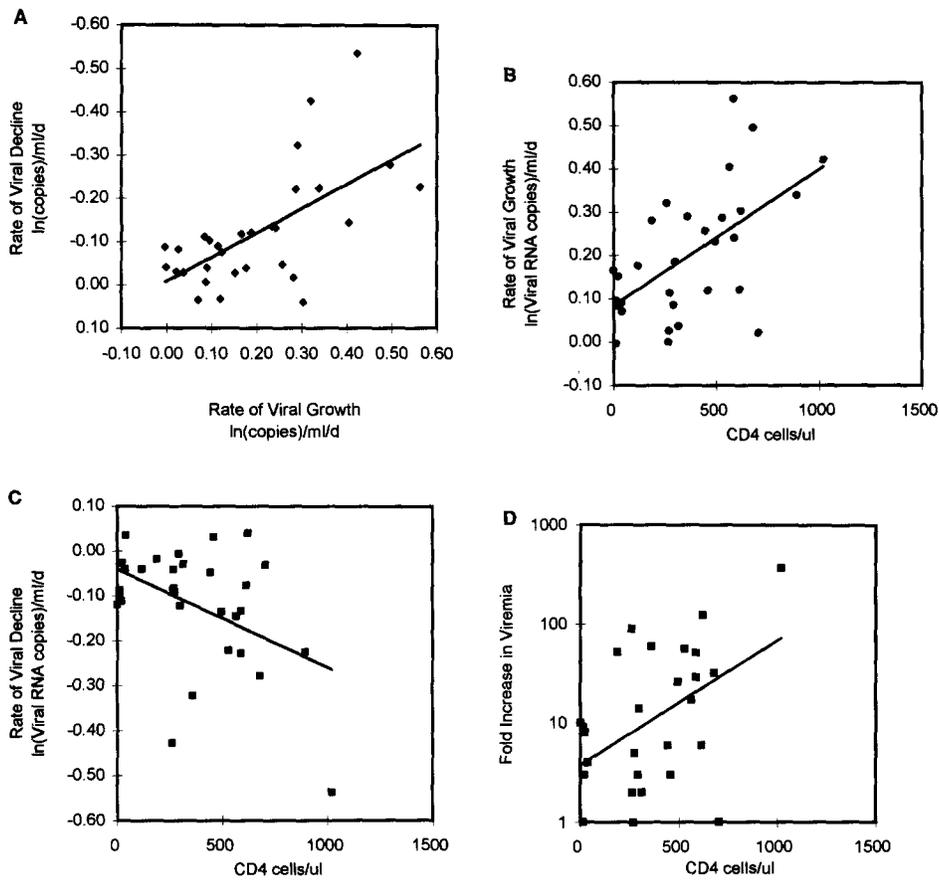


Figure 4. (A) The rate of plasma viral population growth after influenza vaccination and the rate of viral population decline after peak viremia. (B) The rate of plasma viral population growth and prevaccination circulating CD4 lymphocyte count. (C) The rate of plasma viral population decline and prevaccination circulating CD4 lymphocyte count. (D) The fold increase in viral load and prevaccination circulating CD4 lymphocyte count. Each point represents one patient. Rates were calculated as described in the Materials and Methods. The solid lines represent best fits by linear regression. Associations were significant in all comparisons: $P < 0.0002$ (A), $P < 0.0007$ (B), $P = 0.01$ (C), $P = 0.05$ (D).

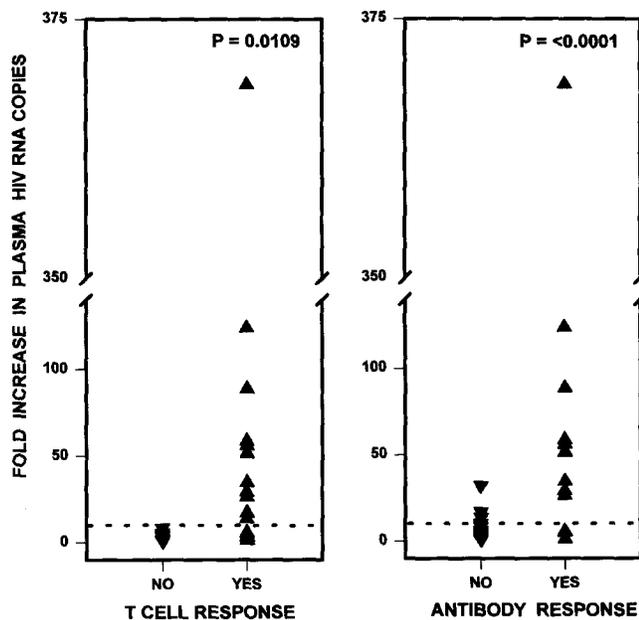


Figure 5. Relationship between an immune response to the influenza vaccine antigens and an increase in plasma HIV RNA copy. Greater increases in plasma HIV RNA copies were observed in subjects who made an immune response to the influenza vaccine antigens (\blacktriangle) compared with those who did not (\blacktriangledown), either as a T cell proliferative response (left) or as a fourfold or greater antibody response (right). P values were calculated by Mann-Whitney rank test.

may predispose the host to infection with specific opportunistic pathogens. Although experimental evidence for this hypothesis has not been pursued previously, certain clinical observations are consistent with its predictions. HIV-1 infection around the time of hepatitis B vaccination increases the risk of chronic hepatitis B virus (HBV) carriage, suggesting that HIV-1 may preferentially target HBV-reactive T cells for infection and depletion (32). In the setting of HIV-1 infection, it is reported that acyclovir therapy in individuals with a prior history of herpesvirus infection, and isoniazid therapy in tuberculin skin test-positive individuals, confer survival advantage and delay disease progression, respectively (33, 34). These observations suggest that reducing the chronic antigenic stimulation caused by these pathogens is beneficial. However, further study is needed to determine whether opportunistic infection-induced virus activation leads to a faster rate of HIV-1 disease progression. If antigen-driven constriction in the repertoire of T cell antigen recognition were demonstrated, this would argue for early antiviral intervention in HIV-1 disease to preserve an intact and diverse repertoire. If loss of the diversity of T cell antigen recognition was irreversible, then T cell repletion by antiviral therapy or lymphokines such as IL-2 in intermediate- or late-stage disease might be of limited use (11, 12, 35).

To better understand the effects of antigen activation in HIV-1 infection, we performed an observational study of HIV-1-infected individuals receiving influenza vaccina-

tion. Large increases in plasma HIV-1 RNA were observed in the majority of patients given the influenza vaccine. Plasma viremia peaked 1–2 wk after immunization and returned to baseline levels in the majority of patients within 4 wk. Virus load increases manifest a time course that parallels the kinetics of the generation of the immune response to the influenza vaccine (36), suggesting that increased plasma viremia occurs as a consequence of vaccine-induced activation of influenza-responsive CD4⁺ T cells (21, 36–39). Furthermore, increases in plasma viremia were higher in those individuals who responded immunologically to the vaccine antigens, compared with those who responded poorly. This result and other recent studies point to virus production arising from continued new rounds of infection of susceptible target cells (11, 12).

The magnitude of increases in plasma HIV-1 levels was surprisingly large, suggesting that they were unlikely to be due to HIV-1 replication in influenza-responsive T cells alone. Rather, the antigen-responsive cells might be necessary to initiate an immune response that is then further amplified by cytokine activation of additional non-antigen-specific cells (40–42). Such amplification of the immune response may increase indirectly the pool of susceptible target cells. Recent evidence suggests that immune activation may indeed increase the population of cells susceptible to HIV-1 infection (Fauci, A., HIV Pathogenesis Meeting, Keystone, CO 1995). Further, we have recently found that vaccination of rhesus macaques around the time of experimental simian immunodeficiency virus infection results in a significant acceleration of the rate of subsequent disease progression, perhaps the result of creation of a larger pool of cells susceptible to virus infection or compromise of the efficacy of the host antiviral immune responses (Staprans, S., and M. B. Feinberg, manuscript in preparation).

Recent kinetic studies using potent antiviral agents to inhibit HIV-1 replication demonstrate that the measured levels of plasma HIV-1 RNA reflect a steady state that is primarily determined by the rate of ongoing virus production, rather than rates of clearance, which were relatively uniform in the patients studied (11, 12). Analysis of the kinetics of plasma viremia after vaccination revealed that the increase in viremia was proportional to the prevaccination CD4⁺ T cell count. Patients with low CD4⁺ T cell counts tended to have slower rates of viral load increases, lower rates of viral decline, and lower fold increases in viremia. Such patterns of viremia might arise from an initially slower but then more prolonged phase of immune activation. Additionally, or alternatively, less efficient immune containment of virus-producing cells might be occurring in these patients. Viremia is both induced and suppressed more quickly in individuals with high CD4⁺ T cell counts. Optimal virus suppression after peak viremia in the healthiest, highest vaccine responder was manifested by a measured virus half-life of 1.6 d. This calculated half-life of plasma virus is similar to the viral clearance rates described in recent studies using potent antiviral drugs (11, 12), suggesting that the antiviral immune response may, in the best of circumstances, contain HIV-1 replication as effectively as the most

active antiviral drugs available. More potent virus suppression in individuals with higher CD4⁺ T cell counts may reflect more active immune destruction of infected T cells, perhaps by cytotoxic T lymphocytes, as opposed to a non-immune-mediated cytolysis of HIV-1-infected cells. Perhaps the measured rate of containment of HIV-1 production after vaccination could be used as a means of assessing the integrity of the antiviral immune response *in vivo*.

Chronic basal antigenic activation of the immune system may contribute to the observed steady-state viral load in HIV-1-infected patients. Alternatively, the steady-state virus load may be due to a non-antigen-driven mechanism involving continued proliferation and replenishment of lost CD4⁺ T lymphocytes (11, 12). It is interesting that, after substantial increases in plasma viremia, almost all of the subjects returned to their previous baseline values of plasma viremia. This suggests that the steady state may result from (a) the number of new cycles of viral infection, which is perhaps determined by the host's immune activation status; and (b) the efficiency of the host antiviral immune response. The state of activation of the host immune system is expected to determine the number of target cells available for HIV-1 infection, whereas the vigor of the host antiviral immune response is expected to determine the number of HIV-1-producing cells present in an infected individual. It is notable that, although a number of the individuals studied displayed low steady state levels of plasma viremia, presumably the result of effective immune containment of HIV-1 replication, they were unable to prevent the surge in virus replication seen after influenza vaccination. The demonstration of more rapid rates of increase in viremia in individuals with higher CD4⁺ T cell counts and the more restricted range of plasma HIV-1 RNA levels seen among study participants at peak viremia after vaccination are consistent with the importance that the size of the pool of susceptible target cells plays in determining the level of ongoing HIV-1 replication in infected individuals.

Antiretroviral therapy initiated at various times before influenza vaccination did not prevent increases in plasma viremia. We cannot distinguish whether this is due to the relative ineffectiveness of the nucleoside analogues available at the time this study was conducted or the presence of drug-resistant viruses in treated patients before vaccination. Treatment with more potent antiviral drugs (11, 12) might be more effective at blunting the activation of HIV-1 replication after vaccination. In addition, future studies of the use of these agents in the setting of vaccination should permit delineation of the relative contributions to increased viremia of the activation of latently infected T cells or the increased availability of susceptible target cells after antigenic stimulation. Further, should antiviral drugs be identified that effectively limit antigen-induced increases in HIV-1 viremia, they might improve the immune responses to vaccination in treated individuals and provide an approach to limit the damage that may potentially result from vaccine- or infection-induced activation of virus replication.

Although we have no evidence to suggest that influenza vaccination was of any clinical detriment to HIV-1-infected

individuals, it is reasonable to be concerned about the substantial, albeit transient, increase in HIV-1 load seen after immunization. Potentially, increased plasma viremia may induce the destruction of either a specific antigen-reactive component of the T cell repertoire, or a more general non-antigen-specific component, or both. However, given the significant activation of virus production that follows a discrete vaccine-induced antigenic exposure, it is likely that the immune activation associated with an actual opportunistic infection may cause even more dramatic stimulation of virus production. These issues must be considered in determining the advisability of particular vaccines. Our additional anecdotal experience suggests that acute *M. tuberculosis* and *P. carinii* infections can cause increased viral load (Staprans, S., and M. B. Feinberg, unpublished observations). Indeed, intercurrent infections occurred in a few of the influenza-vaccinated patients whose plasma viremia levels either did not return to baseline values or who manifested a second, later peak in plasma viremia.

The efficacy of influenza immunization in the HIV-1-seropositive population remains to be determined (43, 44). The current recommendations concerning influenza vaccination of HIV-1-infected individuals stem from analogies made to individuals with other types of immunodeficiency. However, the close link between immune activation and HIV-1 replication suggests that this reasoning may be flawed. Our results, as well as those of others, show that the influenza vaccine is poorly immunogenic in the HIV-1⁺ population, with the lack of immunogenicity being linked to the degree of immune deficiency (18, 45-47). Influenza is not felt to be an important pathogen in HIV-1⁺ individuals, although this population is at increased risk for bacterial infections (*Staphylococcus aureus*, *S. pneumoniae*, and *H. influenzae*) associated with postinfluenza secondary pneumonias (48). Furthermore, an alternative (albeit cumbersome) approach involving prophylaxis with amantadine is available for influenza-exposed people should future studies demonstrate a detrimental impact of influenza vaccination of HIV-1-infected individuals.

Evaluation of the role of vaccination as a prevention strategy for other pathogens such as *S. pneumoniae*, *H. influenzae*, or HBV that are important causes of disease in HIV-1-infected individuals may be quite different (49-51). The multivalent pneumococcal capsular polysaccharide vaccine (Pneumovax) appears to elicit a better immune response than does the influenza vaccine (45, 52). However, like influenza vaccination, pneumococcal vaccination also results in transiently increased levels of HIV-1 replication in many HIV-1-infected individuals (Stevenson, M., personal communication). *H. influenzae B* vaccine also elicits a more potent immune response in HIV-1-infected individuals, although immunization early in disease may be required to obtain levels of antibody response that correlate with protection from infection (53). Prevention of HBV infection through vaccination is clearly important in people at risk for HIV-1 infection. In light of our results and those of others (Stevenson, M., personal communication), it will be important to carefully weigh the risks and benefits of individual vaccines in the context of both pediatric and adult HIV-1 infection.

It is hoped that the recently developed methods to monitor HIV-1 RNA levels in the plasma, including the techniques used in this study, will provide valuable tools to assess the risk of disease progression and the efficacy of antiviral drugs in infected individuals (23, 24, 29). However, little information is available concerning the factors that influence the biological variation of these new assays. The observed changes in plasma viremia after vaccination or infection by pathogens are important to consider in determining the clinical utility of HIV-1 RNA assays, as such perturbations may significantly influence the interpretation of viral load measures.

Further studies will be required to elucidate the possible pathogenic consequences of immune stimulation in HIV-1 infection. It is important to determine whether environmental or vaccine-associated antigenic stimulation results in antigen-specific constriction of the T cell repertoire and progression of HIV-1 disease.

We thank the subjects who participated in this study for their interest in this study and their generous commitment of time to it. Dr. Toby Dynner provided essential assistance in participant recruitment and care. Edwin Charlebois offered valuable guidance with the statistical analyses, and Drs. Rebecca Coleman and Donald Abrams provided important encouragement, advice, and support. We are indebted to Jessica Guthrie, Chris Horton, Birgit Drews, and Marianne Gesner for their expert technical assistance. Drs. Jeff Lifson and Mike Piatak of Genelabs Technologies, Inc. (Redwood City, CA) generously provided reagents and advice concerning the HIV-1 QC-PCR method. The HIV-1 bDNA (Quantiplex HIV-RNA) kits used in this study were generous gifts from the Chiron Corporation, and monovalent influenza vaccine antigens were kindly provided by Wyeth-Ayerst Laboratories.

This work was supported by the J. David Gladstone Institutes (M. B. Feinberg), and an unrestricted grant from Amgen (S. E. Follansbee). S. I. Staprans is a Pediatric AIDS Foundation scholar.

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Received for publication 21 June 1995.

Note added in proof: After submission of this manuscript, a study of the impact of influenza vaccination on HIV-1 RNA expression in peripheral blood mononuclear cells of infected individuals was published (O'Brien, W. A., K. Grovit-Ferbas, A. Namazi, S. Ovcak-Derzic, H. J. Wang, J. Park, C. Yeramian, S. H. Mao, and J. A. Zack. 1995. *Blood*. 86:1082-1089). The conclusions of this study are, for the most part, consistent with our own. However, in contrast to our finding no significant changes in PBMC HIV-1 RNA levels after influenza vaccination (even in persons demonstrating large increases in plasma HIV-1 RNA levels), O'Brien et al. reported transient increases in PBMC HIV-1 RNA levels as measured by a semiquantitative reverse transcriptase PCR assay.

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