Human Immunodeficiency Virus–Type 1 Replication Can Be Increased in Peripheral Blood of Seropositive Patients After Influenza Vaccination

By William A. O'Brien, Kathie Grovit-Ferbas, Ali Namazi, Stanislava Ovcak-Derzic, He-Jing Wang, Julie Park, Christine Yeramian, Si-Hua Mao, and Jerome A. Zack

Despite considerable evidence that cell activation enhances human immunodeficiency virus–type 1 (HIV-1) replication in vitro, there is very little data on the role of immune activation on in vivo HIV-1 replication. In this study, we examined the effect of influenza vaccination on HIV-1 replication in the peripheral blood of 20 study subjects, and in 14 control subjects who did not receive influenza vaccination. Blood was obtained from each subject on three occasions during the month before vaccination and again on three occasions during the following month. Over the study period, there was little change in levels of proviral DNA in peripheral blood mononuclear cells (PBMCs). However, peak PBMC viral RNA levels after influenza vaccination were significantly increased over the mean of prevaccination values. This change was not observed to the same extent in unvaccinated controls. Therefore, this is the first report showing that HIV-1 replication can increase in temporal association with influenza vaccination. Our results suggest that continued immunologic (antigenic) stimulation may result in increased virus load in vivo. To address the appropriateness of influenza vaccination in HIV-infected patients, expanded studies will be required to examine specific and generalized immune responses to vaccination, and differences in patient response based on disease stage.

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HUMAN IMMUNODEFICIENCY virus–type 1 (HIV-1) infection leads to a progressive depletion of CD4+ lymphocytes and acquired immunodeficiency syndrome (AIDS) in a majority of HIV-infected individuals. Although the median time course from acute infection to AIDS is greater than 10 years, the duration of the clinically latent state is highly variable, and factors that govern clinical latency have not been elucidated. After acute infection, there is a viremic phase with widespread distribution of virus. Viremia typically decreases within 2 to 3 weeks, at least in part because of sequestration of virus in lymph nodes as well as development of specific anti–HIV-1 immunity. Although the acute HIV-1 syndrome may be followed by a prolonged period of clinical latency, there does not appear to be virologic latency. High levels of circulating virus particles can still be shown in the plasma of infected asymptomatic individuals by quantitative HIV-1 RNA polymerase chain reaction (PCR) in plasma, as well as in lymphoid tissue. Recently, the dynamic interaction between HIV-1 replication and CD4+ lymphocyte destruction was shown in HIV-infected individuals after administration of potent antiretroviral drugs. Moreover, clinical progression and CD4+ lymphocyte depletion are associated with increases in viral load, as reflected by viral DNA and RNA levels and by infectious HIV-1 titers. Nonetheless, factors responsible for the regulation of virus expression in vivo remain poorly defined.

One potential mechanism of virus induction is immune activation. Activation of CD4+ lymphocytes and mononuclear phagocytes in vitro appears to be important for production of HIV-1. Cellular transcription factors induced after activation of T cells can increase HIV-1 expression. Other studies using cultured primary T cells have shown that, although HIV-1 can bind and enter both quiescent and activated cells, activation is required for completion of reverse transcription, integration, and/or subsequent progeny virus formation. Similarly, activation of mononuclear phagocytes, by adherence to plastic or by cytokine treatment, can dramatically enhance virus production in vitro. Therefore, we hypothesize that immunologic activation may also increase HIV-1 replication in vivo.

To address this issue, we have examined the effect of antigenic stimulation by influenza vaccination on HIV-1 replication in the peripheral blood of HIV-infected patients. Because cell-mediated immunity is important for containing influenza virus infection, HIV-infected patients are thought to be at risk for prolonged and more severe disease, and annual influenza vaccination is specifically recommended. In this report, we show increases in HIV-1 RNA levels in peripheral blood mononuclear cells (PBMCs) temporally associated with influenza vaccination.

MATERIALS AND METHODS

Patients. Study subjects (n = 34) were HIV-1 seropositive and at an intermediate stage of HIV disease at entry to the study with no AIDS-defining illnesses. This study was approved by the Veterans Affairs Medical Center West Los Angeles Internal Review Board for human subjects, and informed consent was obtained for all participants. Subjects were to have CD4+ lymphocyte counts of 100 to 200/mm3, although those having 500/mm3 were eligible if willing to participate. Two patients (01 and 03) were studied in successive years, and are counted twice. Study subjects were self-assigned to the vaccinated (n = 20) or nonvaccinated control group (n = 14), because influenza vaccination was, and still is, recommended for all HIV-infected individuals. Patients were excluded if there was clinical or laboratory
evidence of acute viral hepatitis, active herpes simplex virus infection, pneumonia, or other acute respiratory infection, psychosis, or transfusion within the last 2 months. Patients receiving immunization did not have an allergy to eggs, because vaccine antigens were derived from influenza virus preparations grown in eggs. At each visit, patients were specifically questioned about the presence of fever, cough, rash, cutaneous or respiratory infection, and diarrhea.

**Blood collection and processing.** Blood was collected on three occasions during the month before influenza vaccination, with the third sample obtained on the day of vaccination. These baseline samples were obtained at 7- to 14-day intervals, and spanned a period of at least 3 weeks. Additional blood samples were obtained during the first week, the second week, and at 4 to 6 weeks after vaccination. PBMCs were purified by Ficoll/Hypaque (Pharmacia, Uppsala, Sweden) gradient centrifugation within 3 hours after collection. For PCR analysis, 10^7 cells were lysed in buffer containing 4.7 mol/L urea and stored at -70°C. Nucleic acids were purified simultaneously from all samples for each patient by phenol/chloroform extraction and ethanol precipitation. The remainder of PBMCs were cryopreserved in RPMI 1640 medium, 20% fetal calf serum, and 10% dimethylsulfoxide in the vapor phase of a liquid nitrogen freezer. PBMCs from HIV-negative donors used for plasma infections titer analysis were obtained from Red Cross leukopaks (Los Angeles, CA).

**Quantitative PCR analysis.** Samples from each patient were analyzed in batch on the same test run to avoid interassay variation. Quantitative PCR for human β-globin sequences was performed on samples from each patient to standardize for cell equivalents as a control both for nucleic acid recovery and for amplification efficiency. Sample volumes were then adjusted for subsequent HIV analysis. HIV-specific DNA in each sample was measured by quantitative PCR using 5'-end-labeled long terminal repeat (LTR) R/U5 primers, followed by amplification for 30 cycles at 94°C for 1 minute and 65°C for 2 minutes. HIV-1 copy number was determined by comparison with linearized, cloned HIV-1 DNA standards amplified in parallel as previously described. To measure HIV-1 RNA levels in PBMCs, total nucleic acids for each sample from an equivalent number of cells (standardized by cell number, and subsequently by β-globin DNA content) were treated with RNase-free DNase I at 37°C for 40 minutes to digest the DNA. Purified RNA from 3 × 10^6 cells was amplified using T7th polymerase (Perkin Elmer-Cetus, Norwalk, CT) with reverse transcription in the presence of MnCl2 and 12 pmol of the antisense gag primer LA9 followed by amplification for 35 cycles of amplification using 6 pmol of the γ-32P-labeled sense primer LB19 and 6 pmol cold LA8 added for a total amount of 12 pmol per reaction, under the conditions described above. The absence of HIV-1 DNA was shown by omitting the reverse transcription step in a duplicate sample, and analyzing in parallel. Cell equivalence for RNA analysis was shown by subjecting selected samples to quantitative RT-PCR using β-actin primers. Quantitation was achieved by comparison with amplicons generated from serial dilutions of RNA purified from PBMCs infected in vitro by HIV-1 Liu, CA and subjected to reverse transcription and PCR in parallel. The signal intensity of the amplified product was measured from the autoradiogram by densitometry using an optical imaging system (Ambis, San Diego, CA). Standard curves were generated from the values obtained for β-globin, and HIV-1 DNA and RNA standards, which were used to derive values for experimental samples after interpolation of the standard curve.

**Statistics.** Nonparametric analyses were used to assess change in viral RNA levels. The mean of the three preimmunization RNA measurements for each patient was defined as the baseline RNA level. Relative change in RNA level was defined as the difference between the peak RNA value postimmunization and the mean baseline value, divided by the mean baseline value. The sign rank test was used to evaluate the changes within each study group, whereas the Wilcoxon rank sum test was used to compare the changes between the two study groups.

**RESULTS**

The mean CD4+ lymphocyte count for all study subjects was 301 ± 112/mm³; range, 55 to 547/mm³. Only one subject had a CD4+ lymphocyte count less than 120/mm³; this was patient 01b on his second evaluation. The mean CD4+ lymphocyte count at entry was not significantly different for control and vaccinated patients (337 ± 112 and 277 ± 107, respectively, P = .13). The racial composition of the two study groups was similar; 40% of the study subjects were black, 7% were Hispanic, and the remainder were white. All of the study patients were believed to have acquired HIV-1 infection sexually, none admitted to recent intravenous drug use. All but two of the study patients (04 and 34) were receiving zidovudine therapy.

Patient histories and examinations at 1- to 2-week intervals during the 2-month study period did not show any side effects from vaccination, nor were there any symptoms of acute infections in the study population. Therefore, we do not believe overt infections with bacteria or with heterologous viruses were important confounders during the course of observation. This analysis is critical, because infection may be another potential source of stimulation, and hence, viral induction. In addition, none of the study patients reported blood transfusions or symptoms of allergy or hay fever during the study period.

PBMCs were then analyzed for HIV-1 DNA and RNA levels by quantitative PCR at each time point for each patient. To account for differences in nucleic acid extraction efficiency, each sample for each patient set was standardized for cell equivalence by using quantitative PCR for β-globin gene sequences.

**HIV-1 DNA PCR results for five representative patients are shown in Fig 1. With one exception (patient 04, data not shown), there was no detectable increase in HIV-1 DNA levels on a per-cell basis during the 2-month study period in either the vaccinated or nonvaccinated control group. Of importance, patient 04, a vaccinated subject, had not been receiving zidovudine therapy. Antiretroviral therapy with zidovudine would be expected to inhibit formation of new viral DNA. Patient 26, a nonvaccinated control, exhibited a fall in PBMC HIV-1 DNA levels during the latter half of the 2-month study period, which was roughly matched by a fall in PBMC HIV-1 RNA levels (Fig 2). This higher HIV-1 replication level at the earlier time points may reflect an activation event that immediately preceded the study period.

**HIV-1 RNA was detected in every patient and in most (over 80%) of the individual PBMC samples. Representative HIV-1 RT-PCR experiments from the same five patients in Fig 1 are shown in Fig 2. In contrast with what was observed for viral DNA, there was a significant relative increase in postvaccination HIV-1 RNA levels in PBMC from the 20 patients receiving influenza vaccination (11.6 ± 5.0-fold increase, median 2.7, P < .002). Greater than fourfold increases were seen in 10/20 (50%) of the vaccinated subjects. The peak HIV-1 RNA levels typically occurred at 1 or 2
weeks postvaccination (in 9/10 patients showing greater than fourfold increase), and returned to baseline at later time points. Thus, in most patients, HIV-1 RNA induction was transient. Equivalent increases in peak PBMC RNA levels during the same time frame were not seen in the 14 nonvaccinated controls (2.4–±1.6-fold increase; median, 0.0; \( P = .24 \)), and only 2/14 control patients (14%) had increases greater than fourfold. These data are summarized in Table 1.

As another measure of viral induction, we measured plasma infectious titer by limiting dilution culture analysis\(^{13,14} \) in 15 of the 20 vaccinated subjects, and 4 of the 14 nonvaccinated controls. In this analysis, sequential fivefold dilutions of patient plasma were added to 10\(^5\) phytohemagglutinin (PHA)-stimulated peripheral blood lymphocytes (PBL) in duplicate in 24-well plates, and assayed for p24 production at days 7 and 14. A fivefold or greater increase in infectious plasma virus titer was seen in one of four nonvaccinated controls (patient 15), a patient in whom increases in cellular HIV-1 RNA were also detected. Plasma HIV-1 infectious titer increases of fivefold or greater were also seen in 6 of the 15 vaccinated subjects. Five of these 6 subjects also had increases in cellular HIV-1 RNA, and 1 patient (99) in whom there was a 79-fold increase in cellular HIV-1 RNA, also had a greater than 25-fold increase in plasma infectious virus titer. Thus, increases in plasma infectious virus titer were consistent with results seen for cellular RNA expression.

The peak RNA PBMC level was plotted against the mean baseline levels for vaccinated and nonvaccinated control patients in Fig 3. Comparison of increases between the two groups indicated a trend toward significance of the change in vaccinated patients (\( P = .089 \)). Although there were highly significant changes in PBMC RNA levels after vaccination when the mean value of baseline samples was used as a control for individual patients, there were marked HIV-1 RNA increases at later time points in PBMCs from two nonvaccinated control patients (subjects 15 and 78) that reduced the significance of vaccine-related HIV-1 induction in the overall study population. In retrospect, this type of variation in the control subjects would be expected if immune stimulation by environmental antigens has an influence on virus replication.

We have followed study patients longitudinally to determine the relationship between virologic response to vaccination, and clinical outcome. At more than 3 years mean follow-up, 12 of 20 vaccinated subjects have developed AIDS, and 8 have died. We have indicated the CD4\(^+\) lymphocyte counts at baseline, and 6 months after enrollment in Table 1. Of the 10 vaccinated patients who exhibited a fourfold increase in HIV-1 RNA, 5 had a fall in CD4\(^+\) lymphocyte number at 6 months of 20% or more, and all 5 of these subjects developed AIDS. Moreover, 3 of 5 vaccinated subjects with HIV-1 RNA increases who did not have a decrease in CD4 cell count have not developed AIDS. Finally, 3 of the 10 study subjects who did not show an HIV-1 RNA increase developed AIDS over 6 months, and 2 of these patients had a 20% decrease in CD4 cell count. Therefore, the pattern of virologic response to influenza vaccination does not entirely predict outcome. Other factors appear to be involved in determining the rate of clinical progression.

**DISCUSSION**

These results show that HIV-1 expression in PBMCs markedly increased in one-half of patients during the 2 weeks after influenza vaccination. This appears to be a transient...
induction that generally does not lead to a sustained increase in virus replication. The importance of cell activation for HIV-1 replication has been well shown in vitro. Infection of quiescent PBLs results in an incompletely formed reverse transcript that cannot give rise to infectious virions. Activation of cells with cytokines, lectins, or mitogens either before, or shortly after infection, results in completion of reverse transcription and infectious progeny virions. Chronically HIV-infected cell lines have been used as in vitro models for effects of HIV-1 activation. Cell clones chronically infected with HIV-1 LAV were established from the monocytoïd cell line U937 or the T-cell line CEM (U1 and ACH2 cell clones, respectively), but generally produced low levels of HIV-1.55,56 When these cell lines are activated by cytokines or other agents, viral RNA and progeny virion production is increased in association with increases in a variety of cellular factors.23,37,38 Reporter constructs have also shown increased LTR-driven chloramphenicol acetyl transferase (CAT) expression in cell lines activated with mitogens and/or cytokines.39

There have been other reports suggesting a relationship between immune activation in vivo and induction of HIV-1 replication. Treatment of HIV-1–infected chimpanzees with various vaccine preparations, or adjuvant alone, caused transient increases in numbers of infectious PBMCs.40 Suggestive evidence indicates that both influenza vaccination and influenza virus infection in patients can increase numbers of HIV-1–infected PBMCs.41

We chose to focus our analysis on changes in PBMC HIV-1 RNA, rather than extracellular virus, because of the immediacy of the response to stimulation. Cell activation appears to invoke production of factors that can induce HIV-1 expression, and lead to detectable increases in PBMC HIV-1 RNA.42 We addressed extracellular virus levels using quantitative viral titers in plasma, which yielded similar results. However, plasma viral titer is less sensitive than cellular HIV-1 RNA assays for several reasons. First, infectious assays measure only virus competent for replication in culture. Furthermore, plasma virus assays may be affected by factors elaborated from CD8+ lymphocytes, as well as by antigen-antibody complexing of virions. It will be important to confirm our observation in cells using the newer plasma HIV-1 RNA assays.

Recent studies43,44 have concluded that activation of latently infected PBMCs is not the major source of plasma viremia. Rather, these data suggest that plasma virus originates either from recently infected cells,11 or via trafficking through the lymphoreticular system.40 Therefore, although we show an increase in HIV-1 RNA in circulating PBMCs, activation may result in HIV-1 induction in either the intravascular or the tissue compartments.

We do not believe that the HIV-1 induction observed is a result of the specific response to influenza antigen, because there is a fairly small population of influenza-specific memory cells.42 Furthermore, most patients in our study are not expected to mount a measurable specific response to vaccine.43-45 The HIV-1 induction may be a consequence of a generalized immune response resulting from antigen presentation by macrophages and dendritic cells. This could cause cytokines to be produced that result in both activation of latently infected cells and induction of virus expression, as well as increased replication from infection of newly activated cells.

There are several potential explanations for the transient nature of the HIV-1 induction seen in our study. First, the period of viral induction may be limited by the duration of minor specific T-cell responses to antigen. As influenza-specific T-cell clones return to quiescence or the memory state, cell factors involved in augmenting virus expression are also down regulated.23 Alternatively, stimulation of HIV-infected, influenza-specific T cells may result in deletion of the infected population, and a subsequent decrease in HIV-1 expression. This phenomenon has previously been shown after in vitro antigenic stimulation of PBMCs from HIV-infected asymptomatic individuals, but was not addressed in this study.46 Finally, the fall in virus expression may be related to immune clearance of cells acutely expressing viral antigens. Our studies cannot distinguish between these possibilities. With this in mind, it is unclear why two control subjects exhibited a substantial increase in viral RNA production during the course of the study. Although we cannot document this, it is possible that subclinical infection or other immune stimuli such as an allergic reaction could have led to this induction of viral expression. This is consistent with our hypothesis that other activation events in addition to influenza vaccination can affect HIV-1 replication.

However, there was no evidence in our study for increases in HIV-1 DNA load over the short term. Because most pa-
Table 1. Study Patients Characteristics and Response to Vaccination

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Demographic and clinical data of study patients enrolled over three successive influenza vaccination seasons (1990, 1991, and 1992). CD4 cell counts are expressed per cubic millimeter; values shown are at enrollment, and at 6 months after immunization. Quantitative RT-PCR was performed on PBMC extracts obtained on three occasions during the month before and again at 1, 2, and 4 weeks postvaccination. Relative RNA change is defined in Materials and Methods.

tients were receiving antiretroviral therapy with zidovudine, new viral DNA formation would be inhibited, even in patients exhibiting marked increases in HIV-1 RNA expression. In addition, our assay would not reliably detect increases in HIV-1 DNA of twofold or less, which may still be relevant for clinically important increases in proviral burden. It is also possible that vaccination increased the number of infected cells in compartments not assayed here, such as lymphoid tissues. Although in our study, we did not detect increases in HIV-1 DNA over 2 months, it seems likely that the progressive increases in viral load during the course of HIV disease are a consequence of many such small inductions of HIV-1 replication which occur intermittently over several years. Our study involved only a single immune stimulation event which may be inconsequential to a chronic disease such as AIDS where an infected individual is expected to be exposed to numerous antigenic stimuli. Thus, events that stimulate T cells or macrophages may result in adverse consequences for HIV-infected patients.

In addition to immune-mediated events, another potential cofactor in HIV-1 pathogenesis is infection with heterologous viruses. Coexpression of HIV and either herpes simplex virus or cytomegalovirus genes in T cells, or stimulation of HIV-infected cells by HTLV particles, can result in increases in HIV-1 replication. In addition, a recent study suggests that recurrent herpes simplex virus infection can also lead to marked increases in HIV-1 expression. Furthermore, actual influenza virus infection may lead to a greater level of HIV-1 expression than the transient nature of the increase in viral expression observed here, because of the prolonged nature of the infection. This relationship may hold true for other vaccine-versus-disease combinations; how-
ever, our study does not distinguish which process has the more deleterious effect in HIV-infected individuals. Therefore, it may be necessary to balance HIV-1 induction by influenza vaccination or other immune-based therapies, with the HIV-1 potentiation effects of other acute infections. However, the relative impact of these phenomena on viral load accumulation and disease progression has not yet been delineated, and it appears likely that progressive viral load increases are a result of repeated episodes of immune activation and subsequent bursts of replication that can occur after a variety of stimuli. This study does not attempt to address the appropriateness of influenza vaccination in HIV-infected patients, nor do we suggest that these findings should be generalized to all vaccines.

Our study does suggest that it might be prudent to administer antiretroviral therapy to all individuals receiving immune augmentation therapies to minimize potential increases in viral load. The HIV-1 inductive response to influenza vaccination could not be used in our study to predict clinical course. Immunologic and clinical deterioration ensued over a 3-year period in only half of the vaccinated subjects who exhibited a fourfold or greater increase in HIV-1 RNA expression. Notably, patient 03, who showed vaccine-related HIV-1 induction in 2 different years, has had stable CD4+ lymphocyte counts for over 6 years, and is still without AIDS. Viral strain differences and/or differences in host response may account for the weak correlation between HIV-1 induction and clinical outcome. In addition, the efficacy of vaccination, as well as the HIV-1 response, varies in patients even at similar stages of disease.

Our initial evaluation focused on patients at an intermediate stage of HIV-1 disease. Although there were three patients below 200 CD4+ lymphocytes/mm³ and two above 500/mm³ at study entry, none of the patients had been diagnosed with AIDS. However, it is likely that patients at different stages of disease may exhibit distinct responses. At early stages of disease (patients with over 500 CD4+ lymphocytes/mm³), proliferative responses to antigenic stimulation tend to be more vigorous. These patients also show lower virus load, and more effective antiviral immunologic responses, which may prevent detectable viral induction. However, patients at advanced stages of disease (<200 CD4+ lymphocytes/mm³) show markedly impaired proliferative responses to antigen, and have high background levels of HIV-1 replication. The virologic response to immune stimulation at early or late stages of disease is not well described.

Individual genetic differences are also likely to be important. For example, differences in response to immunization have been shown in patients based on HLA haplotype. Our studies suggest the need for expanded clinical trials to identify subgroups of patients who might be expected to benefit most from vaccination and other immune-based therapies, as well as those in whom vaccination is not helpful and, in fact, may be deleterious. The significance of transient viral induction needs to be assessed using other assays and long-term clinical observation, and in HIV-infected individuals at different stages of disease.

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Human immunodeficiency virus-type 1 replication can be increased in peripheral blood of seropositive patients after influenza vaccination

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