

## EFFECT OF IMMUNIZATION WITH A COMMON RECALL ANTIGEN ON VIRAL EXPRESSION IN PATIENTS INFECTED WITH HUMAN IMMUNODEFICIENCY VIRUS TYPE 1

SHARILYN K. STANLEY, M.D., MARIO A. OSTROWSKI, M.D., JESSE S. JUSTEMENT, B.S., KIRA GANTT, B.A., SUSAN HEDAYATI, B.S., MARGARET MANNIX, R.N., KIM ROCHE, R.N., DOUGLAS J. SCHWARTZENTRUBER, M.D., CECIL H. FOX, PH.D., AND ANTHONY S. FAUCI, M.D.

**Abstract Background.** Activation of the immune system is a normal response to antigenic stimulation, and such activation enhances the replication of human immunodeficiency virus type 1 (HIV-1). We studied the effect of immunization with a common recall antigen on viral expression in HIV-1-infected patients, on the ability to isolate virus, and on the susceptibility to HIV-1 infection of peripheral-blood mononuclear cells (PBMCs) from control subjects not infected with HIV-1.

**Methods.** Thirteen HIV-1-infected patients and 10 uninfected adults were given a 0.5-ml booster dose of tetanus toxoid. Studies were performed to evaluate changes in the degree of plasma viremia, proviral burden, the ability to isolate HIV-1, and the susceptibility of PBMCs to acute infection *in vitro*. Two patients underwent sequential lymph-node biopsies for the assessment of viral burden in these tissues.

**Results.** All 13 HIV-1-infected patients had transient

increases in plasma viremia after immunization, and the proviral burden increased in 11. These changes did not correlate with the base-line CD4+ T-cell counts. The lymph-node tissue also had increases in the proviral burden and viral RNA after immunization. The virus was more easily isolated from PBMCs from nine of the patients after immunization than before immunization. Despite considerable variability in the results, PBMCs from 7 of the 10 normal subjects were more easily infected *in vitro* with HIV-1 after immunization than before immunization.

**Conclusions.** Activation of the immune system by an ongoing antigen-specific immune response to an exogenous stimulus transiently increases the expression of HIV-1 and may enhance the susceptibility of uninfected subjects to HIV-1. (N Engl J Med 1996;334:1222-30.)

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THE critical role of cellular activation in the initiation and propagation of infection with human immunodeficiency virus type 1 (HIV-1) has been well established.<sup>1</sup> A number of *in vitro* studies have demonstrated the importance of activation in the induction of viral expression from infected primary cell lines<sup>2</sup> as well as myeloid and lymphocytic tumor cell lines chronically infected with HIV-1.<sup>3,4</sup> Cellular activation is also important *in vitro* in the acute infection of cells with HIV-1. Resting T cells incubated with HIV-1 generate an incompletely reverse-transcribed proviral DNA that, in the absence of cellular activation, results in an abortive infection.<sup>5,6</sup> In contrast, peripheral-blood mononuclear cells (PBMCs) that have been activated with phytohemagglutinin are readily infected with HIV-1.<sup>5-7</sup>

There is a similar relation between cellular activation and the expression of HIV-1 *in vivo*. Previous studies have suggested that the degree of immune activation in response to simian immunodeficiency virus infection was a factor determining the course of disease,<sup>8</sup> and evidence from studies in both animals and humans indicates that activation of the immune system is associated with altered regulation of HIV-1 in infected patients.<sup>9-14</sup>

The course of HIV-1 infection and progression to the acquired immunodeficiency syndrome (AIDS) appear to be much more rapid in sub-Saharan Africa than in developed countries.<sup>15-18</sup> It has been postulated that the increased rate of disease progression as well as the greater susceptibility to infection after exposure to HIV-1 in sub-Saharan Africa is due at least in part to the persistent

immune activation associated with chronic parasitic infestation and frequent infections with other pathogens.<sup>19</sup>

We undertook the current study to delineate the effect of cellular activation associated with a discrete immune stimulus provided by immunization with a common recall antigen on *in vivo* and *in vitro* expression of virus in HIV-1-infected patients. We also examined the effect of immunization on the *in vitro* susceptibility to acute infection of PBMCs from normal subjects not infected with HIV-1.

### METHODS

#### Patients and Control Subjects

The study was carried out according to a clinical protocol approved by the investigational review board of the National Institute of Allergy and Infectious Diseases. Healthy heterosexual men and women and homosexual men, all of whom were seronegative for HIV-1, were used as control subjects. The patient population consisted of 16 homosexual men seropositive for HIV-1. All participants were asymptomatic; one patient had been given a diagnosis of AIDS on the basis of a total CD4+ T-cell count of less than 25 per cubic millimeter. The participants were either given a 0.5-ml tetanus booster intramuscularly (Wyeth-Ayerst Laboratories, Marietta, Pa.) or mock immunized (three seropositive patients and four seronegative control subjects). Blood was drawn on the day of the injection and approximately 3, 7, 14, 21, 28, and 42 days later and was analyzed as described. Two patients underwent serial lymph-node biopsies before and after immunization. The lymph nodes were from sites unrelated to the site of immunization. All subjects gave informed consent.

#### Processing of PBMCs and Depletion of CD8+ T Cells

PBMCs were obtained by standard Ficoll centrifugation (LSM, Organon Teknika, Durham, N.C.). The samples were depleted of CD8+ T cells by incubation (in a 10:1 ratio) with magnetic beads coated with an anti-CD8 antibody (Dynabeads M450 CD8, Dynal, Lake Success, N.Y.) according to the manufacturer's instructions.

#### Fluorescence-Activated Flow Cytometry

Unfractionated PBMCs and PBMCs depleted of CD8+ T cells ( $0.5 \times 10^6$  to  $1 \times 10^6$  cells) were placed on ice with the appropriate antibodies for 30 minutes, washed, and fixed with 1 percent paraformaldehyde. Antibodies recognizing CD4, CD8, CD3, HLA-DR, CD25,

From the Laboratory of Immunoregulation, National Institute of Allergy and Infectious Diseases (S.K.S., M.A.O., J.S.J., K.G., S.H., K.R., A.S.F.); the Nursing Department, Warren Grant Magnuson Clinical Center (M.M.); and the Division of Cancer Treatment, National Cancer Institute (D.J.S.) — all at the National Institutes of Health, Bethesda, Md.; and Molecular Histology Inc., Gaithersburg, Md. (C.H.F.). Address reprint requests to Dr. Stanley at the National Institutes of Health, Bldg. 30, Rm. 7A03, 31 Center Dr., MSC-2520, Bethesda, MD 20892-2520.

and isotype controls (Becton Dickinson, Mountain View, Calif.) were used. Cells were analyzed with an Epics-C flow cytometer (Coulter, Hialeah, Fla.).

**Measurement of Tetanus-Specific Immune Responses**

Serum samples were examined for tetanus-specific antibodies and PBMCs were assayed for tetanus-specific proliferative responses in vitro according to standard methods.<sup>20,21</sup>

**Isolation of Virus from HIV-1–Infected PBMCs**

Freshly obtained unfractionated PBMCs and PBMCs depleted of CD8+ T cells were cultured in complete medium with 10 percent fetal-calf serum (Hyclone Laboratories, Logan, Utah), 10 U of interleukin-2 per milliliter (Boehringer–Mannheim, Mannheim, Germany), and either no other stimulus or 5 ng of interleukin-4 per milliliter (R & D Systems, Minneapolis). Culture supernatants were harvested every two to three days, and cultures were fed as needed. Serial cultures from a single patient were maintained in a consistent fashion by one laboratory worker. The ability to isolate virus in culture was considered to be increased when the levels of reverse transcriptase in the culture were at least twice those measured before immunization.

**Acute Infection of PBMCs from Control Subjects**

PBMCs from normal subjects were cultured in complete medium with 10 percent fetal-calf serum with or without added interleukin-2 (0, 1, or 10 U per milliliter). After three days, a primary HIV-1 isolate was added to the cultures at a dilution of 10<sup>-2</sup> (25 times the dose required to infect half the cultures) and 10<sup>-3</sup>. Supernatants were harvested every three days, and cultures fed as needed.

**Reverse Transcriptase Assays**

Reverse transcriptase assays were performed according to standard techniques.<sup>22</sup> In general, the results for duplicate supernatants varied by less than 10 percent and the radioactivity of samples labeled with phosphorus-32 (Dupont NEN, New England Nuclear, Newtown, Conn.) was measured with a Betaplate beta counter (model 1205, Wallac, Gaithersburg, Md.).

**Measurement of PBMC-Associated Viral Burden by the Polymerase Chain Reaction**

The polymerase chain reaction (PCR) was performed as previously described.<sup>23</sup> Serial frozen samples of PBMCs from each patient or, in the case of two patients, frozen lymph-node mononuclear cells obtained by excisional lymph-node biopsy were thawed simultaneously and run in the same reaction to avoid assay variability. Two primers (SK38 and SK39) were used to detect HIV-1 proviral DNA; the calculated amount of total DNA in the reaction mix was adjusted by running parallel reactions with HLA primers QH26 and QH27 that amplify the human HLA locus. The amplified product was hybridized with probes end-labeled with <sup>32</sup>P (SK19 and GH64), subjected to electrophoresis on 10 percent polyacrylamide gels, and quantitated on a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.).

**Quantitative Competitive PCR**

Each patient’s frozen plasma samples or pelleted and frozen lymph-node mononuclear cells from the various time points were thawed and run together to avoid assay variability. RNA was purified from stored plasma with the QIAamp blood kit (Qiagen, Chatsworth, Calif.). RNA was extracted from the cell pellets with the QIAshredder and RNeasy mini preps (Qiagen) to homogenize the cells and purify the RNA, respectively. The extracted viral nucleic acids were treated for one hour at room temperature with DNase (Sigma Chemical, St. Louis) to remove any contaminating DNA. Re-

verse transcription, amplification, and quantitation of HIV-1 genomic RNA were performed as described previously.<sup>24</sup> The interassay variability was 2 to 4 percent.

**RESULTS**

**Viral Burden in Plasma and Cells**

Plasma obtained before and at various times after tetanus immunization was analyzed for the presence of HIV-1 RNA. In all 13 immunized patients, significant increases in plasma viremia were detected after immunization; peak viremia occurred 3 to 28 days after immunization, with a mean of 13 days (Table 1). Figure 1 shows the changes in the degree of viremia in 12 of the 13 immunized patients and 2 mock-immunized patients. The magnitude of the increases in plasma viremia ranged from a factor of 2 to a factor of 36; the mean value at base line was 2.18×10<sup>5</sup> copies of HIV-1 per milliliter, as compared with a mean peak value of 7.75×10<sup>5</sup> copies of HIV-1 per milliliter (P=0.001, by the Wilcoxon signed-rank test). Patients with higher total CD4+ T-cell counts had earlier peaks in viremia (day 7), with fairly rapid resolution and return to base-line values (Patients 9, 10, and 12) (Table 1). In contrast, in those with lower CD4+ T-cell counts, plasma viremia tended to occur later after immunization (e.g., Patients 1, 2, and 7) (Table 1). The magnitude of the increase in viremia did not correlate with base-line CD4+ T-cell counts, although the largest increase (by a factor of 36) was seen in Patient 10, with long-term nonprogressive infection. Plasma obtained serially from three mock-immunized patients showed no significant changes in the levels of viremia over a six-week period (Fig. 1E).

One patient of particular interest is Patient 6, who was initially asymptomatic with a CD4+ T-cell count of 336 cells per cubic millimeter. The level of plasma viremia had tripled seven days after tetanus immunization and

**Table 1. Induction of Plasma Viremia after Tetanus Immunization in HIV-1–Infected Patients.\***

PATIENT NO.	CD4+ T-CELL COUNT		VIREMIA†		PROVIRAL BURDEN†	
	BASE LINE	AT PEAK	BASE LINE	PEAK	BASE LINE	PEAK
	cells/mm <sup>3</sup>		copies of HIV-1 RNA/ml		copies of HIV-1 DNA/10 <sup>6</sup> cells	
1	362	255	147,000	437,000 (21)	20,000	80,000 (22)
2	271	138	3,850	10,500 (28)	20	22 (14)
3	350	301	120,000	900,000 (7)	1,000	2,500 (28)
4	389	271	100,000	700,000 (21)	360	1,044 (42)
5	586	482	21,000	45,000 (3)	30	33 (42)
6	336	374	215,000	725,000 (7)	30	75 (42)
7	336	270	75,000	315,000 (21)	13	26 (42)
8	361	274	87,500	241,000 (7)	100	220 (3)
9	497	409	220,000	745,000 (7)	150	360 (3)
10	602	710	10,500	375,000 (7)	50	240 (42)
11	363	211	80,000	225,000 (21)	2,500	5,000 (21)
12	403	690	215,000	725,000 (7)	7	54 (28)
13	8	5	1,550,000	4,970,000 (7)	250	303 (3)
14‡	658	NA	175	NA	ND	NA
15‡	1102	NA	175,000	NA	96	NA
16‡	445	NA	27,500	NA	32	NA

\*NA denotes not applicable, and ND not detectable.

†The day after immunization on which peak values were obtained is given in parentheses for each patient.

‡Mock-immunized patient.

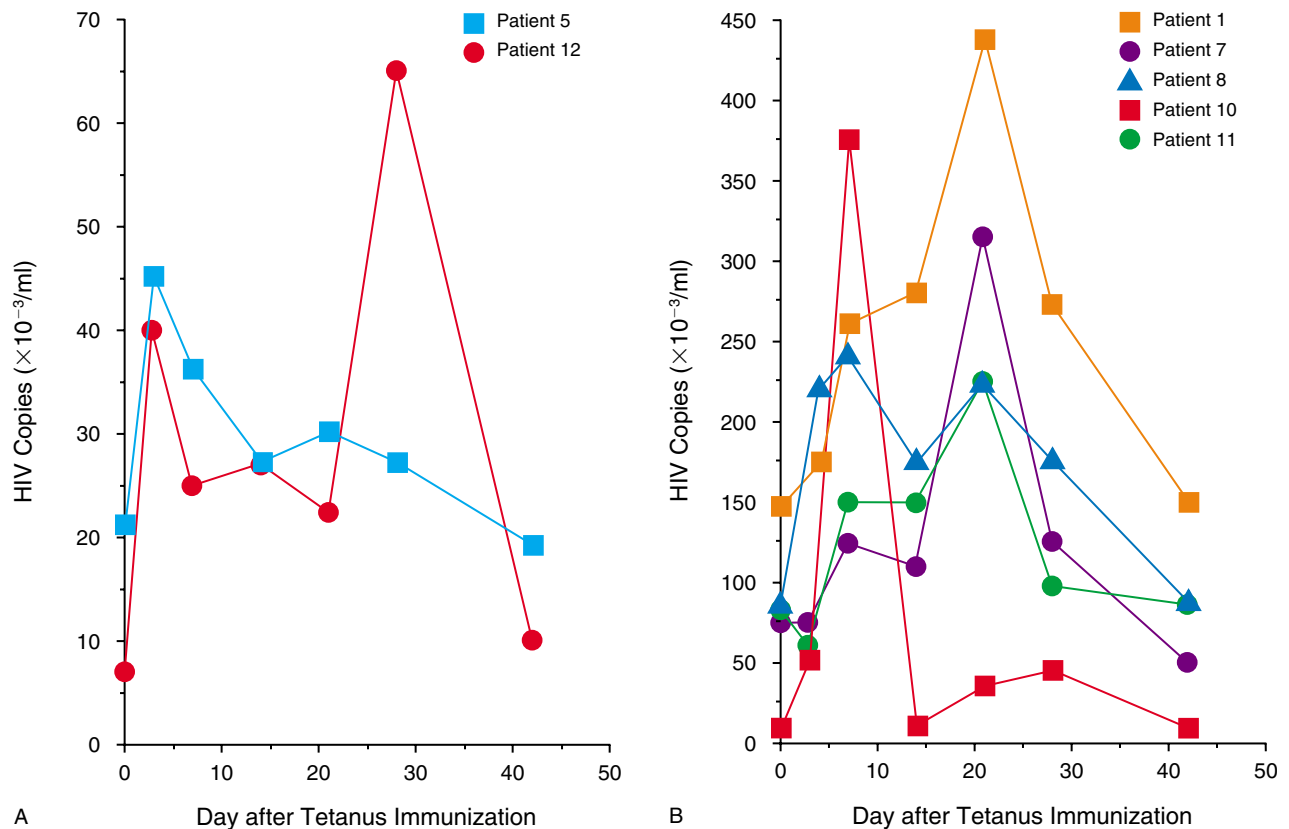


Figure 1. Patterns of Plasma Viremia after Tetanus Immunization or Mock Immunization in 14 HIV-1-Positive Patients (above and on Facing Page).

Patients 14 and 15 were mock immunized.

returned to base line by three weeks (Fig. 2A). However, at a follow-up visit on day 42 he reported a several-day history of hemoptysis and fever and was found to have multiple cavitating lesions on radiographic examination (Fig. 2B). At that time the level of plasma viremia had again increased over base line by a factor of 2.6. After treatment of his pneumonia and resolution of the abnormalities on chest x-ray film, the level of plasma viremia returned to base-line values (Fig. 2A).

Viral burden was also measured in PBMCs obtained at the same times after tetanus immunization as plasma (Table 1). Ten of the 13 immunized patients had increases in the numbers of circulating infected cells, although most of these increases were moderate (ranging from twofold to fourfold). Two patients had substantial increases (Patient 10, by a factor of 4.8; and Patient 12, by a factor of 7.7), and both had long-term nonprogressive infection. There was no correlation between changes in the number of circulating infected cells and changes in the level of plasma viremia. The CD4+ T-cell counts decreased moderately from base-line values at the time of peak viremia in 10 of 13 patients (Table 1); however, in the group as a whole the median decrease was not significant (from 362 cells per cubic millimeter to 274 cells per cubic millimeter;  $P = 0.066$ , by the Wilcoxon signed-rank test).

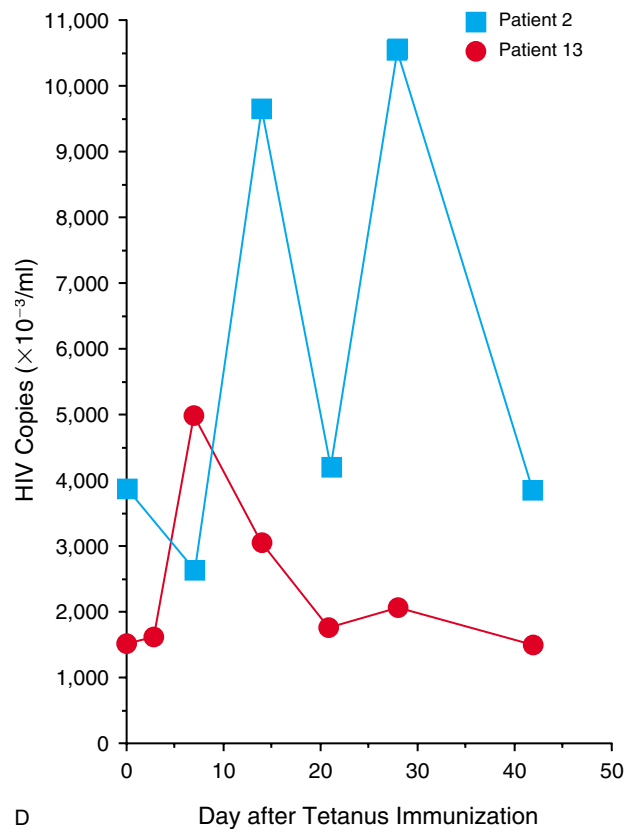
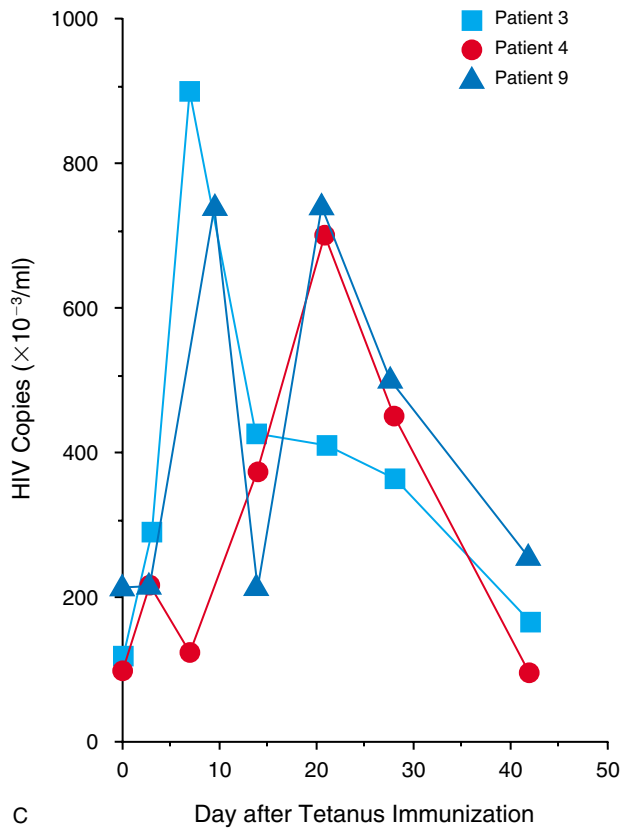
#### Viral Burden in Lymphoid Tissue

Two patients were studied who had had lymph-node biopsies before and after tetanus immunization. The re-

sults for Patient 3 are shown in Figure 3. This patient initially had a hyperplastic lymph node with well-defined germinal centers, and this pattern appeared more pronounced in the biopsy specimen obtained 30 days after immunization (Fig. 3B). In situ hybridization suggested that the trapping of virus within the germinal centers was more pronounced four weeks after immunization; RNA and DNA PCR analysis revealed marked increases in total tissue HIV-1 RNA after immunization and a small increase in the number of proviral DNA copies per million cells (Fig. 3C). Similar results were obtained for Patient 11 (data not shown).

#### In Vitro Isolation of HIV-1

After immunization, it was significantly easier to isolate virus from PBMCs depleted of CD8+ T cells in 9 of 13 immunized patients under minimally stimulated culture conditions (only interleukin-2 and interleukin-4 were added in the absence of mitogens); there were no such increases in the ability to isolate virus from PBMCs from the 3 mock-immunized patients. Figure 4 shows the levels of viral isolation at all time points for Patient 7, who was immunized (Fig. 4A), and Patient 15, who was mock immunized (Fig. 4B), as well as base-line and peak levels for 10 of the remaining 12 immunized patients and 1 additional mock-immunized patient (Fig. 4B, 4C, 4D, and 4E). The level of viral isolation was not considered to be significantly increased over base line in Patient 11 (Fig. 4E) or Patient 12 (Fig. 4F), because of the late isolation of virus on day 21.



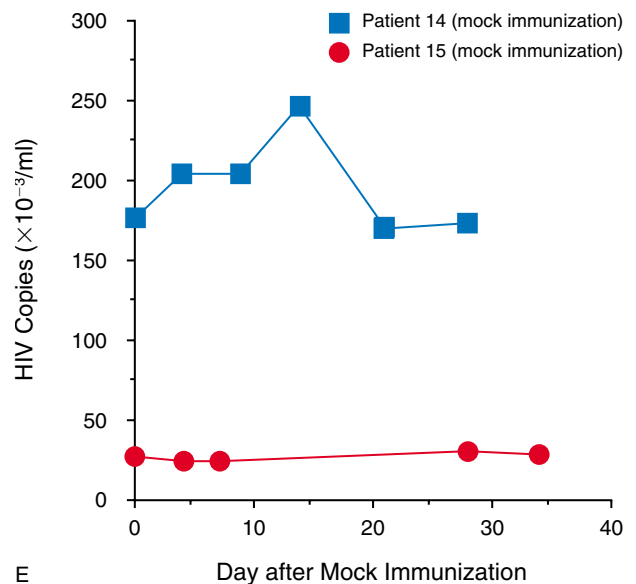
Overall, the in vitro viral production measured on the basis of reverse transcriptase activity in the supernatant increased after immunization by a mean factor of 30 (range, 2.2 to 89). The time of peak isolation (mean, 18 days after immunization) generally occurred after the time of peak viremia for each patient (mean, 13 days after immunization) (data not shown). In most patients, virus could not be isolated from unfractionated PBMCs before immunization (data not shown). After immunization, however, virus was readily isolated from unfractionated PBMCs from Patients 4, 5, 8, and 10.

**In Vitro Acute Infection of PBMCs from Normal Subjects**

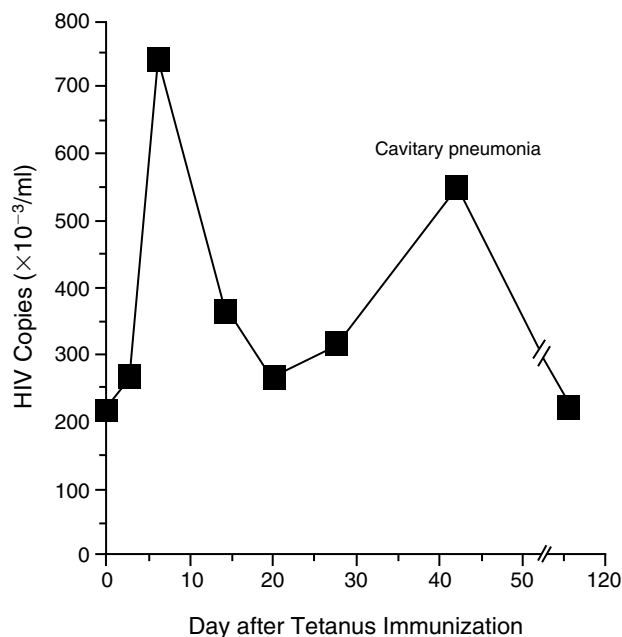
Using a sensitive in vitro culture system in which only interleukin-2 is added as a stimulus, we evaluated the ability of PBMCs from normal subjects to become infected before and after tetanus immunization. Despite considerable interassay variability, cells were more easily infected after immunization than before immunization in 7 of 10 normal subjects. Cells from one subject were somewhat more susceptible to HIV-1 infection within days after immunization but became markedly susceptible three weeks after immunization, when an acute upper respiratory tract infection was present (data not shown). There was considerable variability in the susceptibility to infection of PBMCs from the four mock-immunized normal controls; however, no consistent pattern of increase over time after immunization was seen (data not shown).

**Characterization of the Immune Response**

The expression of two activation markers, HLA-DR and CD25, was measured by fluorescence-activated flow



cytometry before and after tetanus immunization. The base-line level of expression of both markers in uninfected subjects was low (generally less than 5 percent) and increased to a mean of approximately 12 percent of lymphocytes (expressing either marker) (data not shown). In contrast, in HIV-1-infected patients the pattern of immune activation was abnormal. In all but one the expression of CD25 failed to increase from a low base-line level; however, the expression of HLA-DR was elevated at base line in all patients (mean level of expression, 22.1 percent) and increased significantly after



A



B

Figure 2. Pattern of Plasma Viremia in an HIV-1–Infected Patient with Incidental Cavitary Pneumonia after Tetanus Immunization. Cavitary pneumonia developed in Patient 6 six weeks after tetanus immunization (Panel A). Panel B shows the chest x-ray film and computed axial tomographic scan obtained at the time of the diagnosis of pneumonia. A follow-up chest film revealed no abnormalities.

immunization (mean, 38.3 percent; with a mean of more than 44 percent in seven patients). This pattern is consistent with a tetanus-induced activation of the immune response in vivo. Analysis of in vitro proliferation and serum antibody levels revealed that, although cells from all patients proliferated nonspecifically in response to phytohemagglutinin, cells from only one patient (Patient 12) had a 10-fold increase in the tetanus-specific proliferative response in vitro; Patients 2, 7, 9, and 10 had increases of 3-to-6-fold (data not shown). Detectable and significantly increased levels of serum antitetanus antibody developed in five patients (Patients 4, 5, 6, 7, and 10). There was no significant correlation between baseline CD4+ T-cell counts and the magnitude of the increase in plasma viremia, tetanus-antibody response, tetanus-specific proliferation, or expression of activation markers (data not shown). As expected, the uninfected subjects had both tetanus-specific proliferative responses in vitro and increased levels of circulating antitetanus-specific antibodies (data not shown).

### DISCUSSION

The present study clearly demonstrates that cellular activation associated with in vivo immunization with a common recall antigen (in this case, tetanus toxoid) has a major, although transient, effect on the expression of HIV-1 in vivo and in vitro, and immunization appears to increase the in vitro susceptibility to infection of PBMCs from uninfected persons. After immunization the level of plasma viremia markedly increased by a factor ranging from 2 to 36 but returned to base line within six weeks; there was a moderate increase in the proviral burden. In addition, the ability to isolate virus from the PBMCs of infected patients was substantially increased

for a finite period after immunization. The susceptibility of PBMCs from uninfected subjects to acute infection in vitro appeared to be enhanced after immunization as compared with before immunization.

The role of cellular activation in the initiation and propagation of HIV-1 infection of CD4+ T cells in vitro has been well established.<sup>2-7</sup> The validity of these observations in vivo has been demonstrated by several studies in HIV-1–infected humans and chimpanzees as well as in monkeys infected with simian immunodeficiency virus.<sup>8-14,25</sup> Furthermore, reports of the rapid turnover of CD4+ T cells in the presence of high rates of viral replication,<sup>26,27</sup> which implies a heightened level of cellular activation, support the relevance in vivo of the relation between cellular activation and propagation of HIV-1 infection.

It has been proposed that increases in viremia are associated with decreases in circulating CD4+ T cells resulting from direct or indirect virally mediated cytotoxicity.<sup>25,26</sup> We observed that the peak levels of plasma viremia were associated with a small drop in circulating CD4+ T cells in 10 of 13 patients, with no significant differences in CD4+ T cells before and after immunization in the group as a whole. Further studies are ongoing to confirm these observations. Generally, we observed only small increases in the numbers of circulating HIV-1–infected cells (cell-associated viral burden), although three patients had increases that were at least four times the base-line values.

Recent studies suggest that lymphoid organs are the major sites of HIV-1 replication<sup>28,29</sup>; hence, we performed lymph-node biopsies before and 30 days after immunization in two patients. In situ hybridization studies as well as RNA and DNA PCR analysis suggest-

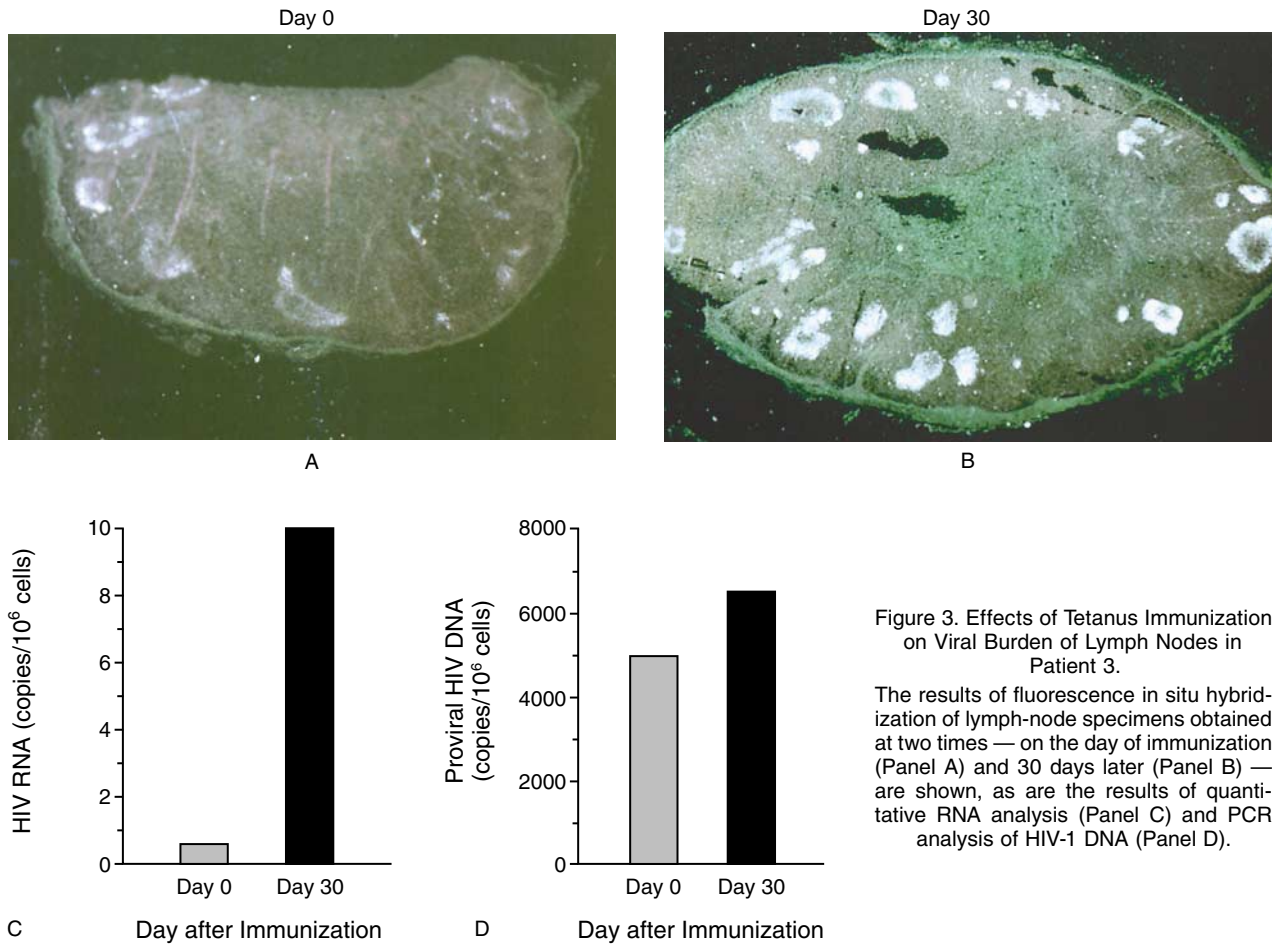


Figure 3. Effects of Tetanus Immunization on Viral Burden of Lymph Nodes in Patient 3.

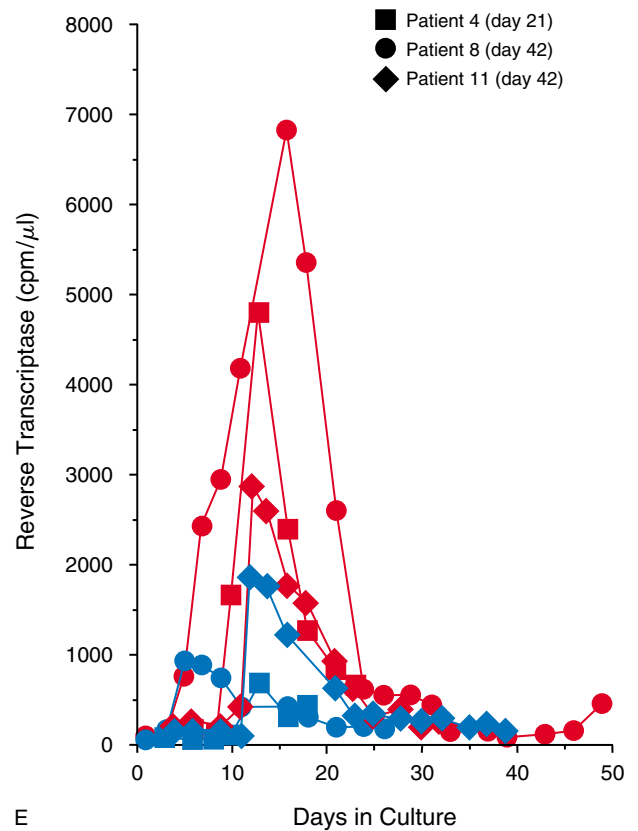
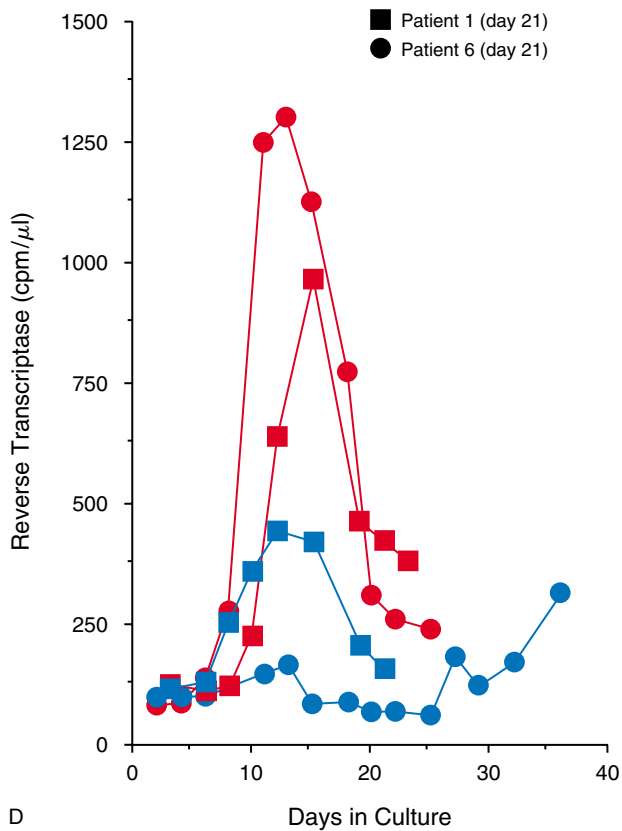
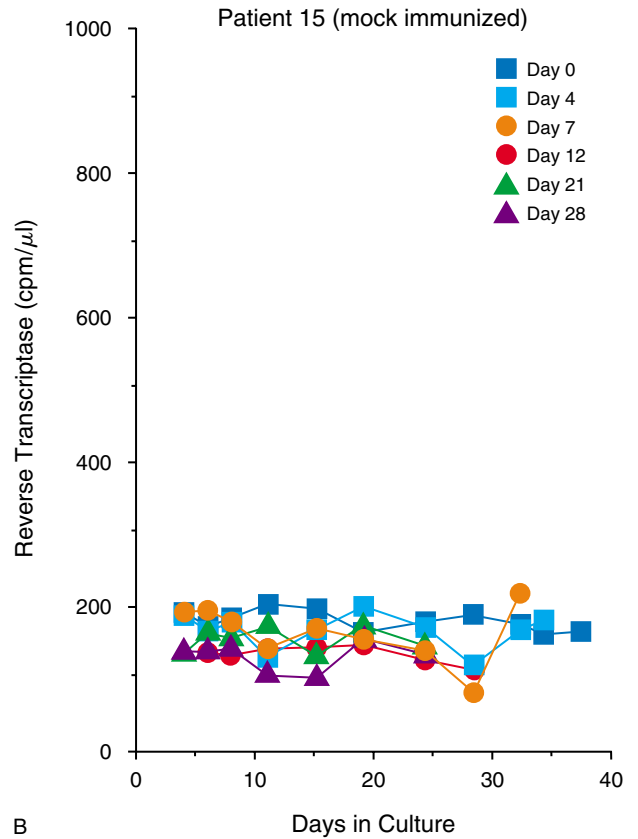
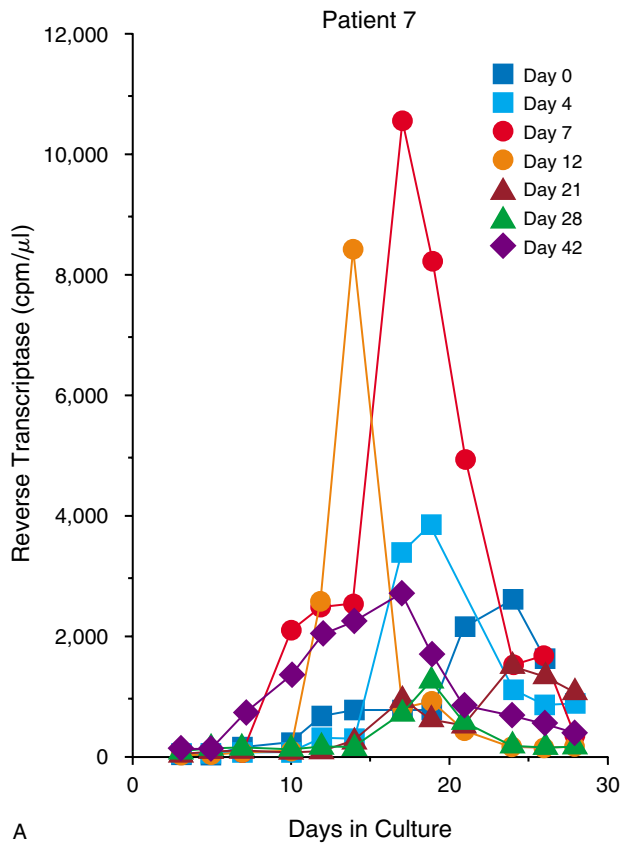
The results of fluorescence in situ hybridization of lymph-node specimens obtained at two times — on the day of immunization (Panel A) and 30 days later (Panel B) — are shown, as are the results of quantitative RNA analysis (Panel C) and PCR analysis of HIV-1 DNA (Panel D).

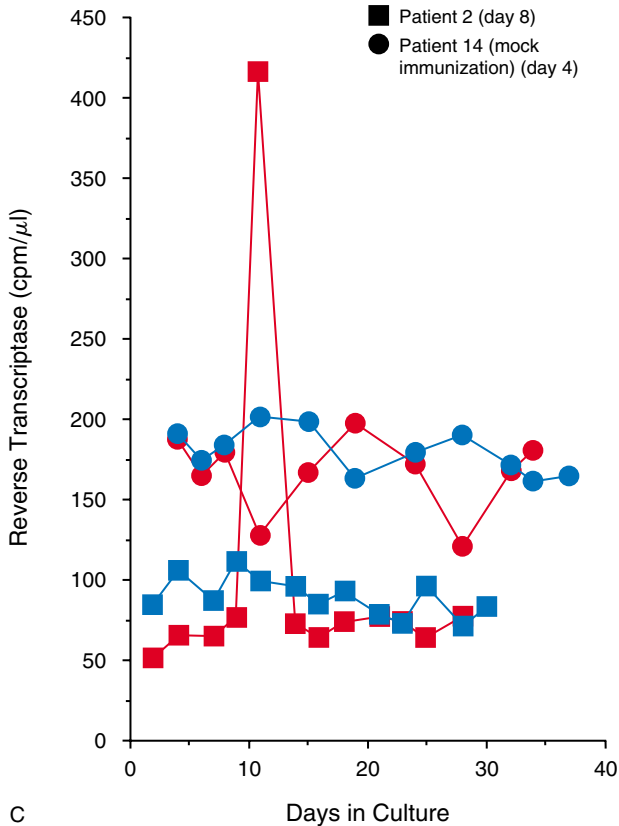
ed that the viral burden increased after immunization with tetanus toxoid. Although a sampling error might explain our findings, the biopsy findings in a single lymph node in an HIV-1-infected patient have been shown to be representative of other lymph nodes.<sup>30</sup> Furthermore, the viral burden has been found to be similar in two lymph nodes taken from the same person eight weeks apart.<sup>31</sup> Given the transient nature of the viremia associated with immunization, it is unclear how long this increase in lymph-node viral burden persists.

The increased ability to isolate virus in vitro after immunization paralleled the increases in plasma viremia. The fact that the ability to isolate HIV-1 in vitro was enhanced even in the absence of antigen indicated that immune activation after immunization was responsible for this phenomenon. Taken together, the increases in plasma viremia and the enhanced in vitro isolation of virus after immunization strongly suggest that immune activation associated with an ongoing immune response may play a part in the pathogenesis of HIV-1 disease. In the case of immunizations, the phenomenon is transient; thus, its pathophysiologic relevance is questionable. However, in cases of ongoing immune activation associated with specific immune responses directed at persistent infections such as parasitic infestations, a sustained increase in the rate of viral replication may have important pathogenic consequences. This possibility is

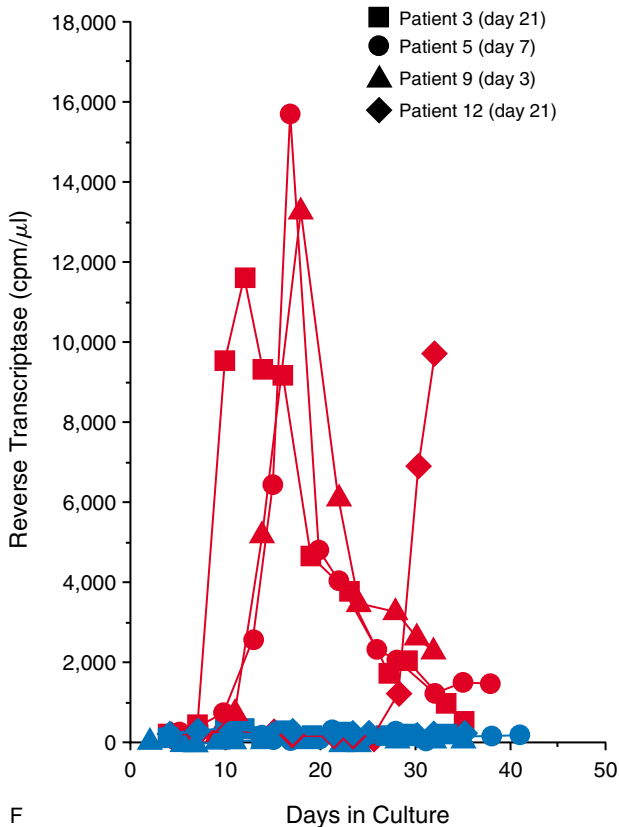
consistent with the hypothesis that the higher rate of disease progression in sub-Saharan Africa is due at least in part to the chronic and persistent immune activation associated with the ongoing immune response to parasitic infestations and other pathogenic microorganisms commonly seen in that region.<sup>19</sup> Along the same lines, the variable but increased susceptibility to in vitro infection with HIV-1 of PBMCs from uninfected subjects after immunization suggests that exposure to HIV-1 during a period of cellular activation associated with an ongoing antigen-specific immune response may increase the probability of infection. This possibility is further supported by our findings in an uninfected subject, whose PBMCs became highly susceptible to HIV-1 infection during an acute upper respiratory tract illness.

An obvious question arises regarding the advisability of immunizations in HIV-1-infected patients in the light of the potential consequences. However, the protection afforded by immunization against a pathogenic organism may outweigh the potential risks from the resulting transient increase in immune activation. It is clear from our experience with Patient 6 that secondary infection, with the development of cavitary pneumonia, was associated with increased plasma viremia. It might be possible to use short-term antiviral therapy to block the transient viremia associated with vaccination. Future studies will address this question. Finally, the pres-





C



F

Figure 4. Pattern of Isolation of HIV-1 in Vitro after Tetanus Immunization or Mock Immunization in HIV-1-Positive Patients (on This and Facing Page).

Data are shown for 11 of the 13 immunized patients and 2 of the 3 mock-immunized patients. No data are shown for Patients 10 and 13 because immunization had no effect on the ability to isolate virus from their PBMCs. All measurements are given for Patient 7 (Panel A) and Patient 15 (Panel B); in the case of the other patients, base-line values are shown in blue and peak values are obtained is given in parentheses for each patient.

ent results may contribute to our understanding of the pathogenesis of HIV-1 disease and serve as the foundation for the development of therapeutic and prophylactic strategies aimed at other microorganisms that might contribute to a state of chronic and persistent immune activation in HIV-1-infected patients.

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**CORRECTION**

**Effect of Immunization with a Common Recall Antigen on Viral Expression in Patients Infected with Human Immunodeficiency Virus Type 1**

Effect of Immunization with a Common Recall Antigen on Viral Expression in Patients Infected with Human Immunodeficiency Virus Type 1 . On page 1227, in Figure 3, panel C, the y-axis should have been labeled from  $0 \times 10^6$  to  $10 \times 10^6$ , not from 0 to 10, as printed. We regret the error. Also, the second part of the legend for Figure 3 should have read, "The results of *35S* riboprobe *in situ* hybridization," not "The results of *fluorescence in situ* hybridization," as printed.