HIV-1 p24 May Persist During Long-Term Highly Active Antiretroviral Therapy, Increases Little During Short Treatment Breaks, and Its Rebound After Treatment Stop Correlates With CD4+ T Cell Loss

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Summary: The dynamics of HIV-1 RNA during structured treatment interruptions (STIs) are well established, but little is known about viral proteins like p24. We studied 65 participants of an STI trial. Before the trial, continuous highly active antiretroviral therapy (HAART) had suppressed their viral load to <50 copies/mL during 6 months. They then interrupted HAART during weeks 1 through 2, 11 through 12, 21 through 22, 31 through 32, and 41 through 52. The p24 was measured by boosted enzyme-linked immunosorbent assay of plasma pretreated by efficient virus disruption and heat denaturation. At time point 0, p24 was measurable in 22 patients (34%), who had maintained a viral load <50 copies/mL for 25.4 months (median, range: 6.2–38.9 months) under HAART. Viral rebounds during 2-week STIs led to a mean p24 increase of only 0.08 to 0.19 log10 (ie, 20%–60%). Pre-HAART viral load and p24 at time 0 independently predicted p24 rebounds during the 4 2-week STIs. The p24 at time 0 and HIV-1 RNA rebound during weeks 41 through 52 independently determined the concomitant p24 rebound. An increase of p24 but not viral load during the first 8 weeks of the long STI correlated significantly with concomitant CD4+ T cell loss. Persisting p24 despite successful HAART may reflect virus replication in reservoirs not represented by plasma viral load and has implications for the concept of therapeutic vaccination.

Key Words: highly active antiretroviral therapy, HIV-1 RNA, viral load, HIV-1 p24 antigen, viral correlates of CD4+ T-cell destruction, treatment interruption

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chronic HIV infection, had been taking 2 classes of drugs (nonnucleoside reverse transcriptase inhibitors excluded) for >6 months, and had presented for >6 months with an HIV-1 RNA level <50 copies/mL in plasma and a CD4+ T-cell count of >300 cells/μL. During the SSITT, the patients underwent 4 cycles consisting of a 2-week treatment interruption followed by an 8-week retreatment phase with the identical drug combination used before the trial. At the fifth and final cycle (week 41 onward) treatment was suspended for >12 weeks if no adverse effects occurred. Weeks 1 through 42 of the trial were used for studying the reappearance of p24 during 5 2-week treatment-free intervals spanning weeks 1 through 2, 11 through 12, 21 through 22, 31 through 32, and 41 through 42. Weeks 39 through 52 were used to study associations between p24, HIV-1 RNA, and CD4+ T cells in a situation of prolonged viral rebound up to a new set point. Patients were included in the present investigation if (1) they had been treated at one of the participating Swiss centers, (2) they had completed the first 4 STIs and started the fifth long STI, and (3) frozen plasma was available for p24 testing. Of the total 133 participants of the SSITT, 65 patients (48.9%) met all criteria; they had a follow-up interval of 44 weeks. One patient each dropped out after weeks 44, 48, and 50. Sixty-two of the patients studied here thus completed the study up to week 52.

**Tests**

CD4+ T cell counts were determined prospectively at weeks 0, 9, 19, 29, 39, 44, 48, and 52. HIV-1 RNA concentration in plasma determined prospectively by the ultrasensitive Amplicor Monitor version 1.5 test, with a quantification limit of 50 copies/mL, was available from weeks 0, 2, 9, 12, 19, 22, 29, 32, 39, 42, 44, 46, 48, 50, and 52. HIV-1 RNA concentration before initiation of HAART was available from the Swiss HIV Cohort Study database. Concentrations of cell-associated unspliced HIV-1 RNA and proviral DNA at week 0 were available from study files and had been determined batchwise as described elsewhere.14,25 The p24 was tested retrospectively at the same time points at which HIV-1 RNA had been tested. All remaining plasma samples, stored at −70°C, were analyzed after heat denaturation by a signal amplification–boosted enzyme-linked immunosorbent assay (ELISA).6 All specimens of a given patient were tested on the same ELISA plate, thus minimizing test-associated variation. The HIV-1 p24 Ag Ultra kit (Perkin Elmer Life Sciences) was used according to the manufacturer’s instructions except for the following crucial modification of sample pretreatment: 100 μL of plasma was added to an Eppendorf tube containing 50 μL of a mixture comprising 30 mM of Tris/HCl pH 7.2, 450 mM of NaCl, 1.5% Triton X-100, 1.5% deoxycholic acid (sodium salt), 0.3% sodium dodecylsulfate, and 10 mM of ethylenediamine tetra-acetic acid (EDTA), mixed well and left for 10 minutes at room temperature. After further dilution with 450 μL of the kit’s prediluted Dissociation Solution, the sample was boiled for 5 minutes on a Technne dry heat block preheated to 100°C. After cooling down to room temperature, 250 μL of the mixture was tested in duplicate wells according to the manufacturer’s instructions. Concentrations were deduced by end point reading from a 3-fold dilution series of standards extending from 170 to 10,000,000 fg/mL. Frozen aliquots of prediluted standards were used in all experiments to reduce intertest variation. Reactivity in the test was related to a quantitative cutoff based on 4 negative controls run on every assay plate. As in earlier studies this cutoff equaled the mean plus 3 standard deviations (SDs) of these negative controls.9,17,18

**Evaluation and Statistics**

For statistical analysis, the StatView 5.0 program for Macintosh (SAS Institute, Cary, NC) was employed. The Wilcoxon signed-rank test and paired t tests were used for analyzing differences in concentrations. The Spearman rank correlation, Pearson correlation, and multivariate linear regression analysis were used for assessing correlations. All concentrations were evaluated as log10-transformed values. HIV-1 RNA <50 copies/mL was set to 50 copies/mL. The p24 concentrations were entered as calculated based on the standard curve, irrespective of whether reactivity was above or below the cutoff. This is justified because the concentrations directly represent the recorded absorbance readings (ie, experimentally determined values) and because many of the statistical analyses remained significant even when only p24 concentrations below the cutoff were considered, as detailed in the Results section. HIV-1 RNA was always above the limit of quantification when analyzed together with p24.

**RESULTS**

We investigated 65 patients, 39 men and 26 women, with a median age of 40 years (range: 22–68 years), who had received antiretroviral treatment for 27.0 months (median, range: 8.5–44.6 months) and had presented with <50 copies/mL of plasma viremia for 24.7 months (median, range: 6.2–42.8 months). On entry into the study, these patients interrupted their individual treatment during weeks 1 through 2, 11 through 12, 21 through 22, 31 through 32, and, finally, for a 12-week period beginning with week 41. p24 persists during long-term suppressed viremia. At time point 0 of the study, whereas HIV-1 RNA was <50 copies/mL in all patients, p24 reactivity was above the cutoff in 22 (34%) patients. At time point 0, p24 exhibited no dependence on the pre-HAART viral load, the total duration of HAART or of viremia <50 copies/mL under HAART, the gain in CD4+ cells while receiving HAART, the concentrations of peripheral blood mononuclear cell (PBMC)–associated proviral DNA or HIV-1 RNA at time point 0, age, or gender (all correlations P > 0.15). Positive results were found in patients who had maintained <50 copies/mL during a median time of 25.4 months (range: 6.2–38.9 months). The fraction with p24 reactivity above the cutoff remained similar at the end of each retreatment period (ie, at weeks 9, 19, 29, and 39) (Fig. 1).

**p24 Increases Little During Structured Treatment Interruptions**

During all 5 STIs, the concentrations of p24 rose little compared with HIV-1 RNA (Fig. 2). During the first STI, mean viral RNA rose from <50 copies/mL or <1.7 log10 to 2.36 log10 (ie, by >0.66 log10). In contrast, mean p24 increased by only 0.19 ± 0.37 log10. For weeks 12, 22, 32, and 42, the respective mean increases of p24 were 0.11 ± 0.36, 0.19 ± 0.31,
The resulting ratios by which the increases of HIV-1 RNA exceeded those of p24 were 5.2, 5.6, 4.7, and 4.8 (average: 4.6 ± 1.0). Despite their low magnitude, the p24 increases during successive 2-week STIs were statistically significant in cycles 1, 3, and 4 (Wilcoxon test, \( P < 0.0001 \) for all 3 cycles) and weakly significant for the first 2 weeks of cycle 5 (\( P = 0.042 \)). The p24 increase during the first STI but not the subsequent short STI correlated significantly with the corresponding HIV-1 RNA peak (Spearman \( \rho = 0.393; \ P = 0.005 \)). Concentrations of p24 at many time points off therapy, namely, at weeks 12, 22, 32, 44, 46, 48, and 50, correlated significantly with those of HIV-1 RNA before initiation of HAART (Spearman \( \rho = 0.26–0.48; \ P = 0.0003 \) to \( P = 0.05 \)). Multivariate linear regression analysis indicated that p24 at time point 0 and pre-HAART viral load were independent predictors of p24 levels at weeks 2, 12, 22, and 32 (Table 1A). The influence of p24 at time point 0 was always stronger than that of the pre-HAART viral load.

The p24 also increased less during the long STI than did the HIV-1 RNA (see Fig. 2). Mean HIV-1 RNA rose rapidly from <50 copies/mL (<1.7 log\(_{10}\)) at week 39 and reached a peak of 3.6 log\(_{10}\) at weeks 46 through 48. The mean increase amounted to >1.9 log\(_{10}\) (i.e., >80-fold). For p24, the overall mean increase was approximately 0.5 log\(_{10}\) (3.2-fold). The relative increase of p24 was at least 25-fold lower than that of HIV-1 RNA. Nevertheless, p24 and HIV-1 RNA concentrations correlated significantly at all time points (Spearman \( \rho: 0.33–0.61; \ P = 0.001 \) to \( P = 0.05 \)). The p24 levels during this period were, however, also markedly influenced by the p24 concentration at time point 0. Indeed, p24 at time point 0 was correlated significantly or at least showed a trend of correlation with all subsequently measured p24 concentrations up to and including week 52 (Spearman \( \rho: 0.27–0.53; \ P < 0.001 \) to \( P < 0.07 \)). This even remained the case if only patients with a p24 level below the cutoff at time point 0 were analyzed, indicating that even p24 levels below the cutoff represented true concentrations rather than chance values.

We investigated by multivariate linear regression analysis how p24 concentration at different time points during the final long STI was influenced by the concomitant concentration of HIV-1 RNA and p24 at time point 0 (see Table 1B). All models were adjusted for age, gender, and other parameters listed at the bottom of the table. The p24 at time point 0 was...
Concomitant pre HAART viral load was independently associated with the CD4+ count at week 39.

Further changes in CD4+ cell counts during weeks 44 through 48 were associated with CD4+ cell counts at week 44 and with p24 at week 44 and its change during weeks 44 through 48 (model 2). These factors remained significant when the entire interval spanning weeks 39 through 48 was examined (model 3). No significance was found for the CD4+ cell count gained during uninterrupted HAART or for any variable listed at the bottom of the table. Similar findings were seen for the change in nontransformed CD4+ cell numbers (not shown). None of the factors considered were associated with CD4+ cell counts at week 48.

The Rebound of p24 After Treatment Stop Correlates with CD4+ T Cell Loss

As seen from Figure 2, there was a noticeable drop in CD4+ cell counts during weeks 39 through 44 (−0.094 \log_{10}; Wilcoxon signed-rank test, \(P < 0.0001\)). Subsequent changes lacked significance despite considerable individual change. The pattern of CD4+ cell count changes in the patients studied here was in accordance with that seen in the whole SSITT.19

We investigated whether CD4+ cell counts or their changes during the final STI correlated with the respective concentrations or changes of the 2 viral markers. CD4+ cell counts at weeks 44, 48, or 52 did not correlate with HIV-1 RNA at any time point. There was also no correlation of the CD4+ cell count changes between any of these time points with the concomitant or preceding HIV-1 RNA changes (not shown). In contrast, there were multiple correlations between CD4+ cell counts and p24 at different time points (Table 2).

Multivariate linear regression analysis was used to investigate the factors that determined the CD4+ cell count changes in the final STI (Table 3). The models investigate how the CD4+ cell count changes during 3 time intervals were influenced by the concentrations of CD4+ cells and p24 at the beginning of the respective interval and by the p24 changes during the interval. We also investigated a reported effect of the gain in CD4+ cells during uninterrupted HAART preceding the study on the CD4+ cell loss after treatment cessation.26 All models were also adjusted for gender, age, and time of HIV-1 RNA <50 copies/mL during uninterrupted HAART. The CD4+ cell count drop observed during weeks 39 through 44 was independently associated with the CD4+ count at week 39 and the p24 increase during weeks 42 through 44 (model 1).

### TABLE 1. Correlates of P24 Concentrations Achieved During the 5 Structured Treatment Interruptions (STI). Multivariate Linear Regression Analysis

<table>
<thead>
<tr>
<th>Short Treatment Interruption</th>
<th>1st STI (Week 2) (n = 59)</th>
<th>2nd STI (Week 12) (n = 57)</th>
<th>3rd STI (Week 22) (n = 53)</th>
<th>4th STI (Week 32) (n = 61)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre HAART Viral Load [(\log (c/mL))]</td>
<td>0.166 (0.029 to 0.302) 0.0181</td>
<td>0.189 (0.070 to 0.307) 0.0025</td>
<td>0.191 (0.078 to 0.304) 0.0014</td>
<td>0.144 (0.019 to 0.270) 0.0246</td>
</tr>
<tr>
<td>p24 at day 0 [(\log (fg/mL))]</td>
<td>0.561 (0.247 to 0.875) 0.0007</td>
<td>0.556 (0.290 to 0.822) 0.0001</td>
<td>0.658 (0.412 to 0.903) 0.0001</td>
<td>0.546 (0.250 to 0.842) 0.0005</td>
</tr>
<tr>
<td>Concomitant HIV-1 RNA [(\log (cc/mL))]</td>
<td>0.150 (0.051 to 0.255) 0.0063</td>
<td>0.176 (0.046 to 0.306) 0.0118</td>
<td>0.274 (0.103 to 0.446) 0.0031</td>
<td>0.251 (0.011 to 0.491) 0.0413</td>
</tr>
<tr>
<td>p24 at day 0 [(\log (fg/mL))]</td>
<td>0.764 (0.474 to 1.053) 0.0001</td>
<td>0.667 (0.329 to 1.006) 0.0009</td>
<td>0.799 (0.459 to 1.140) 0.0001</td>
<td>0.705 (0.228 to 1.181) 0.0058</td>
</tr>
</tbody>
</table>

All models also adjusted for age, duration of ART, duration of pre-ART viral load, cell-associated DNA at study entry, cell-associated HIV-1 RNA at study entry, CD4+ at study entry.

### TABLE 2. Correlation of CD4 Counts and P24 During the Final Treatment Interruption

<table>
<thead>
<tr>
<th>Variables</th>
<th>Pearson's Correlation (R)</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4+ [(\log_{10} (cells/\mu L))] at week 44</td>
<td>p24, week 44</td>
<td>50</td>
<td>−0.254</td>
</tr>
<tr>
<td>CD4+ [(\log_{10} (cells/\mu L))] at week 48</td>
<td>p24, change wks 39–44</td>
<td>49</td>
<td>−0.282</td>
</tr>
<tr>
<td>CD4+ [(\log_{10} (cells/\mu L))] at week 52</td>
<td>p24, week 48</td>
<td>48</td>
<td>−0.431</td>
</tr>
<tr>
<td>CD4+ [(\log_{10} (cells/\mu L))] at week 52</td>
<td>p24, change wks 39–48</td>
<td>47</td>
<td>−0.415</td>
</tr>
<tr>
<td>CD4+ [(\log_{10} (cells/\mu L))] at week 52</td>
<td>p24, change wks 42–44</td>
<td>43</td>
<td>−0.379</td>
</tr>
</tbody>
</table>

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the parameters was regarding the CD4+ cell count change in the interval during weeks 48 through 52. Thus, during the first 8 weeks of the final STI, p24 was a significant correlate of CD4+ count change.

**DISCUSSION**

We used an STI trial to study the dynamics of HIV-1 p24 on treatment withdrawal. We found that (1) p24 in plasma persisted in one third of patients after a median of 2 years of stably suppressed viremia (see Fig. 1), (2) levels of persisting p24 were affected little during short STIs (see Fig. 2), (3) the concentrations of the persisting p24 and rebounding RNA determined the concentration of the p24 rebound after final treatment cessation (see Table 1), and (4) the rebounding p24 correlated inversely with the CD4+ cell count changes seen during the first 8 weeks of the final STI (see Tables 2, 3).

STI was originally proposed as an “autovaccination” concept by which the immune system of patients with suppressed virus replication under HAART would be boosted by brief intervals of virus replication. Results of STI studies have shown, however, that virus control in the absence of HAART is rare.\(^{19}\) The autovaccination concept implies that patients successfully treated by HAART lack viral antigen and that sufficient viral immunogen is produced during STI. Our findings suggest that neither expectation was met in the SSITT. Although more sensitive tests may detect residual HIV-1 RNA in a similarly high proportion\(^{29-32}\) the p24 detected in our patients cannot be the structural p24 contained in the few virus particles present in plasma.\(^{33}\) Based on an estimate of 2000 to 4000 p24 molecules contained in 1 virus particle,\(^{34}\) 6150 to 12,500 particles (or 12,500–25,000 copies of viral RNA) are needed for 1 pg of particle-associated p24. The mean concentration of p24 at week 0, approximately 1 pg, exceeded the maximum of particle-associated p24 present in a plasma with <50 copies/mL by 3 orders of magnitude.

Ultrasensitive assays have shown that in contrast to HIV-1 RNA and reverse transcriptase, most of the p24 present in chronically infected patients cannot be pelleted and is located outside virus particles as immune-complexed protein or free protein of essentially unknown origin.\(^{35}\) The absence of a correlation between p24 at time point 0 and the pre-HAART viral load, the duration of HAART, or the duration of viremia <50 copies/mL in this study suggests that this p24 does not represent protein remaining from the time before HAART. Lack of correlation with PBMC-associated HIV-1 RNA or DNA at time point 0 also suggests that PBMCs are not a major source. The p24 may instead be derived from ongoing virus replication in reservoirs not represented by HIV-1 RNA in plasma. Persisting viral protein may also have implications for the concept of therapeutic vaccination in such patients. The persistence of viral protein after prolonged HAART is in agreement with the increased CD8+ T-cell activation state of such patients compared with controls negative for HIV-1.\(^{36,37}\) It is tempting to speculate that this residual immune activation is attributable to p24 and other persisting viral antigens. This hypothesis is testable but exceeds the scope of this paper.

With regard to the dose aspect of the autovaccination concept, it is evident that the virus rebounds during short STIs, although leading to sizable increases in HIV-1 RNA,\(^{7}\) had a limited additive effect, with average p24 increases of only 0.08 to 0.2 log\(_{10}\) (ie, 20%–60%) (see Fig. 2), thus rendering a pronounced strengthening of the antiviral immune response unlikely. Indeed, during the 4 short STIs, the CD8+ T-cell
responses in half of the patients of the SSITT appeared with a delay or not at all, although many patients exhibited stronger responses after the final STI, when more virus was produced. Thus, the short STI may indeed have produced a suboptimal dose of viral immunogen.\textsuperscript{38} It would be of interest to analyze the CD8+ T-cell responses individually with respect to the p24 doses of viral immunogen.\textsuperscript{38} It would be of interest to analyze Thus, the short STI may indeed have produced a suboptimal responses after the final STI, when more virus was produced.\textsuperscript{38} The discrepancies are intrapatient correlation between p24 and HIV-1 RNA; it did present small study. Our findings contrast sharply with those of an influence of persisting viral protein on the possibility of an influence of persisting viral protein on the to be of relevance to therapeutic vaccination studies. The persistence of viral protein would also seem to be of relevance to therapeutic vaccination studies. The possibility of an influence of persisting viral protein on the outcome of such studies should be assessed.

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REFERENCES


**APPENDIX**