Dynamics of the pool of infected resting CD4 HLA-DR− T lymphocytes in patients who started a triple class five-drug antiretroviral regimen during primary HIV-1 infection

Sanjay UC Sankatsing1,2*, Rieneke ME van Praag1,2, Ronald P van Rij4, Ronald Rientsma4, Suzanne Jurriaans3, Joep MA Lange1,2, Jan M Prins2 and Hanneke Schuitemaker4

1National AIDS Therapy Evaluation Center (NATEC), 2Department of Internal Medicine, Division of Infectious Diseases, Tropical Medicine and AIDS, and 3Department of Human Retrovirology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands
4Sanquin Research and Landsteiner Laboratory, Amsterdam, The Netherlands

*Corresponding author: Tel: +31 205 664 479; Fax: +31 206 918 821; E-mail: s.u.sankatsing@amc.uva.nl

Starting standard antiretroviral therapy within 10 days after the onset of a primary HIV-1 infection cannot prevent the establishment of a reservoir of HIV-1-infected memory CD4 T cells. Here we studied the reservoir of HIV-1-infected memory CD4 T cells in four patients who started a triple class, five-drug regimen during primary HIV-1 infection. There was a strong correlation between the proportion of productively infected CD4 HLA-DR− T lymphocytes and plasma HIV-1 RNA levels (r=0.852; P<0.001) during the first 24 weeks of therapy. Within 45 weeks of treatment, in three of the four patients the proportion of productively infected CD4 HLA-DR− T lymphocytes was reduced below the level of quantification. In the fourth patient the cellular reservoir remained quantifiable. In two patients who stopped therapy 44 weeks after initiation an immediate rebound of the plasma HIV-1 RNA level and the proportion of productively infected CD4 HLA-DR− T lymphocytes occurred.

In conclusion, initiation of a potent five-drug, triple class regimen during primary HIV-1 infection does not result in virus-specific immune control upon discontinuation of therapy after 44 weeks. Therefore, longer or even stronger suppression of viral replication might be necessary to achieve this goal in primary HIV-1 infection.

Introduction

Replication-competent HIV-1 can readily be isolated from resting memory CD4 T lymphocytes of HIV-1-infected individuals with well suppressed plasma HIV-1 RNA levels due to triple or quadruple antiretroviral drug combinations [1–3]. This cellular viral reservoir has been shown to decay with a mean half-life varying between 6 and 44 months [4–6]. The difference in decay rate was attributed to differences in drug efficacy, since individuals who experienced episodes of plasma viraemia had a slower decay rate of their cellular reservoir [7]. The continuous presence of HIV-1 cDNA episomes, which are indicative of recent infection events, indeed pointed to ongoing HIV-1 replication during treatment with a triple or quadruple antiretroviral drug regimen [8]. Even therapy with a triple class, five-drug regimen does not result in clearance of the cellular viral reservoir in chronically infected patients, as we demonstrated recently [9].

The initiation of highly active antiretroviral therapy (HAART) during primary infection may result in the establishment of HIV-specific immune responses [10,11]. However, initiation of a three-drug regimen as early as 10 days after the onset of symptoms of a primary HIV-1 infection could not prevent the establishment of a pool of infected resting CD4 HLA-DR− T lymphocytes, despite successful suppression of plasma viraemia [12]. Here we studied the CD4 HLA-DR− T lymphocyte reservoir in individuals who received a triple class, five-drug antiretroviral regimen during primary HIV-1 infection.

Materials and methods

Patients

Four patients who presented with a symptomatic primary HIV-1 infection [13] started a triple class, five-drug regimen (stavudine, lamivudine, abacavir, nevirapine, indinavir with low-dose ritonavir [14,15]) within 11 (patient 023), 14 (patient 024), 28 (patient 025) and 56 (patient 026) days after the onset of symptoms. Baseline values for each patient are presented in Table 1. To determine size and dynamics of the cellular reservoir for replication-competent HIV-1, we performed quantitative microcultures using graded numbers of highly enriched CD4 HLA-DR− T lymphocytes as we did in a previous study [9]. We monitored...
the proportion of CD4 HLA-DR– T lymphocytes harbouring replication-competent HIV-1 at days 0, 4 and 7 after the start of therapy and at weeks 4 and 24 in all patients and thereafter two to four times during a follow-up period varying between 90 and 117 weeks.

Patient 24 stopped therapy after 45 weeks at his own request. Additional samples for the measurement of CD4 HLA-DR– T lymphocytes harbouring replication-competent HIV-1 were taken from this patient at week 52, 95 and 113 after starting initial therapy. This patient restarted therapy at his own request at week 95. Patient 26 stopped therapy after 42 weeks at his own request and additional samples for the measurement of CD4 HLA-DR– T lymphocytes harbouring replication-competent HIV-1 were taken from this patient at week 71 and 90. At week 90 therapy was restarted because of a low CD4 T-cell count.

The Medical Ethics Committee of our hospital approved the study and written informed consent was obtained from all patients.

Assays

**Plasma HIV-1 RNA**

HIV-1 RNA concentrations in EDTA plasma were measured every 8 weeks using the NucliSens HIV-1 QT assay (Organon Teknika, Boxtel, The Netherlands). During the first 16 weeks of antiretroviral therapy HIV-1 RNA was measured more frequently. When RNA concentrations decreased to below 50 copies/ml, an initial input volume in the assay of 2 ml plasma was used combined with an adaptation of the protocol allowing ultrasensitive detection, resulting in a lower quantification limit of 5 copies/ml [12].

**Quantification of CD4 HLA-DR– T cells harbouring replication-competent HIV-1**

Replication-competent HIV-1 was isolated from CD4 HLA-DR– T lymphocytes according to a modification of the method described by Finzi et al. [1] that we described in detail previously [9]. Briefly, peripheral blood mononuclear cells (PBMCs) were isolated from fresh EDTA blood via Ficoll density gradient centrifugation. CD4 T cells were isolated by positive selection using anti-CD4-coated magnetic beads (MACS CD4 multisort kit, Miltenyi biotec, Bergisch Gladbach, Germany). CD4 T cells were released from the beads and subsequently incubated with anti-HLA-DR-coated magnetic beads. HLA-DR+ cells were obtained by depletion of the magnetic beads that had bound HLA-DR+ cells. The median purity of the CD4 HLA-DR– T cell fraction was 94% (IQR 89–96%). CD4 HLA-DR– T cells were stimulated in vitro with irradiated allogeneic healthy donor PBMCs in the presence of phytohaemagglutinin (PHA) and recombinant human (rh)IL-2. After overnight stimulation, cells were washed and co-cultivated with 2 to 3 days PHA-stimulated PBMCs obtained from at least two healthy donors in rhIL-2-supplemented Iscove’s medium under limiting dilution conditions in 96- or 24-well tissue culture plates for 21–28 days. Every week, supernatant was harvested for analysis of virus production by an in-house p24 ELISA. The proportion of cells that in culture produced progeny replication-competent virus (productively infected cells) was estimated, assuming a Poisson distribution using a Maximum Likelihood approach as described [16].

As lymphocytes are already strongly activated in vivo during primary HIV-1 infection [17], we decided to perform virus isolation both directly from patient cells without an in vitro activation step and after overnight stimulation according to the protocol described above.

**Statistical analysis**

To calculate decay slopes of plasma HIV-1 RNA and lymphocytes harbouring replication-competent HIV-1, linear regression was performed on a plot of the natural logarithm of viral load (that is, plasma HIV-1 RNA or cellular infectious load) over time. The slope of the regression line was used to calculate the half-life of plasma HIV-1 RNA or lymphocytes harbouring replication-competent HIV-1, assuming first order decay characteristics.

The association between plasma HIV-1 RNA and replication-competent HIV-1 from CD4 HLA-DR– T lymphocytes in patients with a primary infection was analysed using Pearson correlation on log transformed

<table>
<thead>
<tr>
<th>Patient</th>
<th>Baseline plasma HIV-1 RNA (copies/ml)</th>
<th>Baseline CD4 T cell count (cells/mm³)</th>
<th>HIV-1 RNA*</th>
<th>CD4 HLA-DR– T cells*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Decay slope (per day)</td>
<td>t₁₀₀ (days)</td>
</tr>
<tr>
<td>023</td>
<td>340,000</td>
<td>700</td>
<td>-0.446</td>
<td>1.6</td>
</tr>
<tr>
<td>024</td>
<td>25,000</td>
<td>670</td>
<td>-0.439</td>
<td>1.6</td>
</tr>
<tr>
<td>025</td>
<td>480,000</td>
<td>410</td>
<td>-0.318</td>
<td>2.2</td>
</tr>
<tr>
<td>026</td>
<td>310,000</td>
<td>480</td>
<td>-0.6</td>
<td>1.2</td>
</tr>
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</table>

*Decay slopes were calculated during the first week of therapy.
data obtained on days 0, 4, 7 and weeks 4 and 24 after start of therapy. If no replication-competent HIV-1 could be isolated from CD4 HLA-DR– T lymphocytes, the lower detection limit was calculated and used as input for these analyses. Paired T test was used to compare the frequency of productively infected CD4 HLA-DR– cells with and without overnight stimulation. SPSS (release 7.5.2, SPSS, Inc. Chicago, Ill., USA) was used for all statistical analyses.

Results

Four patients who presented with symptomatic primary HIV-1 infection were initiated on a five-drug regimen within 11–56 days after the onset of symptoms. Following initiation of therapy plasma HIV-1 RNA levels declined rapidly (Figure 1). During the first 4 weeks of therapy the number of CD4 HLA-DR– T lymphocytes that produced progeny virus in vitro was significantly lower (P<0.06) when cells were first stimulated before co-cultivation with target cells (Figure 2A). For further analyses, only data obtained with unstimulated CD4 HLA-DR– T lymphocytes were used (Table 1, Figure 1, 2B).

At the moment therapy was initiated, a high proportion of CD4 HLA-DR– T lymphocytes already contained replication-competent HIV-1 (Figure 1). The decline of productively infected CD4 HLA-DR– lymphocytes completely paralleled the decline of HIV-1 RNA in plasma (Figure 1). The decay of productively infected CD4 T lymphocytes followed a biphasic pattern, with a rapid initial decay in the first week of treatment, followed by a much slower decay thereafter. The half-life of the plasma HIV-1 RNA and productively infected HLA-DR– cells during this first week of therapy were in the same order of magnitude (Table 1), indicating a similar turnover of productively infected HLA-DR– cells and plasma HIV-1 RNA. In agreement with the observed similar kinetics, we observed a strong correlation between the proportion of productively infected CD4 lymphocytes and plasma HIV-1 RNA levels (r=0.852; P<0.001, Figure 2B). After 24 weeks of

Figure 1. Changes in plasma HIV-1 RNA levels and the proportion of CD4 HLA-DR– T lymphocytes containing replication-competent HIV-1 (IUPM) during a five-drug regimen initiated in four patients soon after the onset of a symptomatic primary infection.
therapy, the proportion of CD4 HLA-DR– T lymphocytes containing replication-competent HIV-1 was 1, 0.2, <0.4 and <0.3 infectious units per million cells (IUPM) for patients 023, 024, 025 and 026, respectively.

In patient 23 the amount of CD4 HLA-DR– T lymphocytes containing replication-competent HIV-1 decreased during the first 24 weeks of treatment but remained stable above the lower limit of quantification (LLQ) during 116 weeks of therapy (Figure 1). In patient 24, at week 45 the proportion of CD4 HLA-DR– T lymphocytes containing replication-competent HIV-1 was <0.3 IUPM, at which time the patient stopped all therapy. Within 6 weeks the proportion of CD4 HLA-DR– T lymphocytes containing replication-competent HIV-1 increased to 12 IUPM (Figure 1). In patient 25 the amount of CD4 HLA-DR– T lymphocytes containing replication-competent HIV-1 dropped below the LLQ in week 24 and remained below the LLQ during 98 weeks of follow-up (Figure 1).

In patient 26 the amount of CD4 HLA-DR– T lymphocytes containing replication-competent HIV-1 increased to 12 IUPM and in week 90 it had dropped to 36 IUPM. At that time the patient restarted therapy because of a decline of the CD4 T cell count (Figure 1).

Discussion

In the four patients with a primary HIV-1 infection a high proportion of productively infected CD4 HLA-DR– T lymphocytes was already observed at 11–56 days after onset of symptoms, confirming previous reports that the cellular reservoir for HIV-1 is established very early in infection [12,18]. After initiation of a five-drug regimen, plasma HIV-1 RNA as well as the proportion of HIV-1-infected CD4 HLA-DR– T lymphocytes declined rapidly, with comparable kinetics, which is in agreement with other observations that the plasma and the cellular viral load are closely related [19].

The rapid decline of the cellular reservoir after start of therapy in primary infection provides evidence for a very rapid turnover of the so-called resting HIV-1-infected CD4 HLA-DR– T lymphocytes. Recently, a biphasic decay of CD4 HLA-DR– T lymphocytes harbouring replication-competent HIV-1 during treatment has been described [18,20]. In these studies, the initial rapid decline was attributed to the loss of the labile pre-integration complex of HIV, followed by a much slower decline of cells harbouring integrated proviral DNA. The existence of labile incomplete proviral DNA products of reverse transcription or full-length proviral DNA species arrested in the cytoplasm has indeed been demonstrated in quiescent T cells that lack the activation state required for completion of the viral replication cycle [21,22]. Mathematical modeling of the decline of plasma HIV-1 RNA after initiation of therapy showed that a biphasic RNA response to therapy may be due to continuous dissemination of HIV-1 during therapy rather than a reflection of the independent decay of two virus producing cell populations with different life-spans [23]. In line with this, the comparable

Figure 2A. Relation between the proportion of in vitro overnight (o/n) stimulated and unstimulated CD4 HLA-DR– T lymphocytes that produce progeny replication-competent HIV-1 in vitro

![Figure 2A](image)

Figure 2B. Correlation between levels of plasma HIV-1 RNA and the proportion of CD4 HLA-DR– T cells harbouring replication-competent HIV-1 at days 0, 4 and 7, and at weeks 4 and 24 after start of treatment in patients with primary HIV-1 infection

![Figure 2B](image)

Prior to culture, CD4 HLA-DR– T lymphocytes were not stimulated in vitro. Symbols indicate the time points after start of treatment: circles day 0, squares day 4, triangles up day 7, triangles down week 4, diamonds week 24. Linear regression was performed on combined data from all patients and all time points.
kinetics of plasma viral RNA and replication-competent HIV-1 in HLA-DR- cells after initiation of therapy in our study may thus imply that CD4 HLA-DR- cells are continuously infected after initiation of therapy and that true latency of this viral reservoir may be doubted.

We previously demonstrated that a five-drug, triple class regimen resulted in a stronger long-term suppression of plasma HIV-1 RNA compared to a clinically successful treatment with standard therapy [24].

In our study, initiation of intensive therapy with five drugs during primary infection reduced the cellular HIV-1 reservoir below the LLQ in three of the four patients (patients 24, 25 and 26) within 45 weeks (respectively, 45, 24 and 24 weeks) after starting therapy. Unfortunately, discontinuation of antiretroviral therapy in patient 24 (after 45 weeks of treatment) and in patient 26 (after 42 weeks of treatment) resulted in an immediate rebound of viral RNA in plasma and replication-competent HIV-1 in resting HLA-DR- cells to baseline levels. Apparently, during this intensive treatment with up to six drugs for 40 weeks, virus-specific immune control, which could have resulted in contained virus replication after discontinuation of therapy, was not established. It cannot be excluded that the almost complete suppression of virus replication due to this intensive regimen has prevented viral antigen exposure and consequently the generation of a virus-specific immune response [25]. In that case, less optimal suppression of virus replication may be more favourable.

Alternatively, a longer duration of therapy with more potent antiretroviral drugs [26] may achieve a better suppression of low-level viral replication even in the cellular reservoir of primary HIV-1-infected patients.

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References


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