Apoptosis and proliferation kinetics of T cells in patients having experienced antiretroviral treatment interruptions

Pierre-Marie Roger1*, Jacques Durant1, Michel Ticchioni2, Philippe Halfon3, Jean-Philippe Breittmayer2, Christelle Brignone2, Sylvie Chaillou1, Brigitte Dunais1, Pierre Dellamonica1 and Alain Bernard2 for the Groupe d’Etudes Niçois Polyvalent en Infectiologie (GENPI)

1Service des Maladies Infectieuses et Tropicales, Hôpital de l’Archet 1, Centre Hospitalo-Universitaire de Nice, BP3079, 06202, Nice cedex 3; 2Unité INSERM 343, Centre Hospitalo-Universitaire de Nice, Hôpital de l’Archet 1, BP3079, 06202, Nice cedex 3; 3Alphabio, Marseille, France

Keywords: apoptosis, proliferation, HIV infection, therapeutic interruption

Introduction

There are two main goals in structured antiretroviral treatment interruption (STI): firstly, to let wild-type drug-sensitive virus re-emerge, and secondly, to stimulate CD8+ T cells by the expected viral rebound. The first potential indication for STI is based on the usual absence of drug resistance in wild-type strains of virus. Thus, patients having experienced several failures of antiretroviral treatments, as a result of multiple drug resistance, may benefit from treatment holidays. The second potential indication for STI is based on the observation of lower CD8 + T cell activity by the expected viral rebound and long-term undetectable viral load. Precise indications for STI are not fully delimited, but it could result both in an increased immune response against HIV, and limitation of drug toxicity. Whatever the goal of STI, it leads usually within a few weeks to viral rebound and immunological changes. It appears, comparing different reports, that patients with a mildly advanced immune defect may have a severe decrease in CD4+ T cells. To the best of our knowledge, mechanisms of CD4+ T cell decrease occurring during STI currently are not determined.

Basically, HIV replication might induce both proliferation and/or apoptosis of T cells. The aim of this study was to determine the kinetics of T cell subpopulation changes, T cell apoptosis and mononuclear cell proliferation in patients who undergo STI.

Methods

This is a prospective study involving 10 patients enrolled after informed consent. As the result of acquired highly resistant strains, these patients experienced multiple antiretroviral treatment failures.

Sample collection

Six blood samples were collected from the date of STI, and thereafter at weeks 2, 4, 6, 8 and 12. Viral load determination, T cell subset count and apoptosis were determined on fresh cells, and peripheral blood mononuclear cell (PBMC) proliferation was measured on defrosted cells.

Quantification of plasma viraemia

HIV-1 RNA plasma levels were measured using a commercially available assay, according to the manufacturer’s instructions (Amplicor

*Corresponding author. Tel: +33-04-92-03-54-52; Fax: +33-04-93-96-54-54; E-mail: roger.pm@chu-nice.fr
ART, antiretroviral treatment.

HIV-1 Monitor Test, Roche Laboratories), with a threshold sensitivity of 40 copies/mL.

**Flow cytometry analysis**

The mouse monoclonal antibodies (mAbs) specific for human surface antigens used for identification of T cell subpopulations were purchased from TEBU Laboratory (Le Perray en Yvelines, France) and included: CD45RA-PE, CD45RO-PE and CD38-PE. Antibodies CD3-FITC, CD4-PC5 and CD8-PE are produced in our laboratory. Lymphocyte subsets were determined on freshly isolated blood using monoclonal antibodies to CD3, CD4 or CD8, CD45RA or CD45RO, and CD38 in triple staining. Following acquisition of 5000 CD3+ lymphocytes, analysis was performed using a FacStar flow cytometer and CellQuest software (Becton Dickinson, Mountain View, CA, USA).

**Apoptosis assay**

T cell subsets and apoptosis of T cell subsets were determined using cytofluorometry, as previously reported.15 Hoechst 33342 dye, which detects programmed cell death (PCD)-associated DNA alterations, was used to quantify apoptotic cells. For the apoptosis assay, PBMC (5 × 10⁶ cells/mL) were cultured overnight, in the medium alone or with agonistic mAbs to Fas/CD95, 30 ng/mL final dilution (Euromedex, Souffel-Weyersheim, France), in 48-well plates. In our experiments (data not shown), this latter resulted in 100% death of Jurkat cells. For each sample, 5000 lymphocytes with a specific phenotype were analysed with CellQuest software in a FACSVantage cytometer (Becton Dickinson). Each condition was tested in duplicate. Remaining cells were frozen in a mixture containing 80% (RPMI + 50% FCS) + 20% dimethyl sulfoxide.

**Proliferation assay**

The proliferation capacity of PBMC after STI was tested using frozen cells. Cells were thawed rapidly and washed twice in medium; cell viability was evaluated by Trypan Blue exclusion. Proliferation was assessed using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA). This is a colorimetric method measuring the production of soluble products, which is proportional to the number of viable cells.16,17 Experiments were performed, following the manufacturer’s instructions, using an ELISA plate reader (iEMS Reader, LabSystem, Cergy-Pontoise, France). In preliminary experiments, we had determined that the optimal conditions for observing an absorbance increase at 490 nm were 50 000 cells per well, with a 4 day culture duration (data not shown). Each time-point was tested in triplicate. Wells in triplicate, containing medium and reagents but without cells, served as a negative control, to determine the background absorbance, which was subtracted from other absorbance values.

**Genotypical resistance**

HIV resistances were determined as previously described.18 Briefly, sequencing of the major part of the reverse-transcriptase gene (nucleotides 25–230) and the entire protease gene were performed on plasma HIV-1 RNA extracted from patients’ plasma samples. The viral RNA was retrotranscribed into complementary DNA, and subsequently amplified by single tube PCR using the TruGene HIV-1 assay (Visible Genetics, Toronto, Canada), following the manufacturer’s instructions. Bidirectional DNA sequencing of the amplification products was performed with a sensitive sequencing method (CLIP, Visible Genetics). Each sequencing reaction was loaded into a MicroGene Clipper sequencer (Visible Genetics). The sequence of each sample was compared to a database of known drug-resistance mutations, to find out which mutations were present in the HIV-1 RNA. Classification of the mutations, associated or not with decreased drug sensitivity, was established according to the consensus statement on antiretroviral drug-resistance testing.19

**Statistical analysis**

In order to show the impact of STI on apoptosis and proliferation of mononuclear cells, we have calculated the percentage of variation from baseline for each patient as follows: percentage of variation = [apoptosis or proliferation level at W(x) − apoptosis or proliferation level at baseline]/apoptosis or proliferation level at baseline. Statistical analysis was based on Wilcoxon’s non-parametric rank test. A P value of <0.05 was considered significant. The whole analysis was performed on Statview F-5 software (Abacus, CA, USA).

**Results**

Ten patients were included in this prospective study over 12 weeks, during which no adverse event, such as acute community and/or opportunistic infection, was observed. There were six male and four female patients, with a median age of 40 years (range: 35–53). The risk factors for HIV acquisition were drug addiction in three, heterosexuality in four and homosexuality in three. All the patients presented with multiple antiretroviral treatment failures. Their characteristics are described in Table 1.
Longitudinal analysis of virological and immunological parameters in 10 patients who benefited from 12 weeks of STI. (a) The kinetics of plasma viral load and T cell subset counts. Plasma viral load was determined using a commercially available assay, and CD4+ and CD8+ T cell subset percentages were measured by a cytofluorometric technique. The percentage of variation from baseline is represented for the three parameters. As expected, viral load increased significantly and in contrast both T cell subsets decreased. However, only a decrease in CD8+ T cells was statistically significant. The kinetics of naive and memory CD4+ T cells, CD8+ CD38+ T cells and naive CD8+ CD45RA+ T cells over 12 weeks of STI. These T cell subpopulation counts were determined by triple staining and a cytofluorometric technique. A decrease in both CD4+ T cell subsets, as well as an increase in double-positive CD8+ CD38+ T cells and naive CD8+ T cells, was observed; the latter subset increased, but only for a transient period. A significant difference, compared with baseline value, according to Wilcoxon’s rank test is indicated as: *P<0.05; **P<0.01.

A significant increase in plasma viral load from baseline to week 12 was observed: median 4.12 (1.77–5.27) to 5.34 (4.86–6.13) log10 copies/mL (P = 0.0005). The CD4+ T cell count decreased by a mean of 80 cells/mm3 from baseline to week 12 [median: 319 (41–1209) to 258 (20–501), P = 0.09]. In the same period, CD8+ T cells decreased by a mean of 139 cells/mm3 from baseline to week 12 [median: 927 (477–3700) to 888 (437–2948), P = 0.043]. The kinetics of viro-immunological changes over 12 weeks are shown in Figure 1(a).

Three-colour flow cytometry analysis showed that among CD3+ positive cells, a similar relative decrease in naive and memory CD4+ T cell counts, as determined by the expression of CD45RA+ or CD45RO+, was observed from baseline to week 12: –44% (–74 to +25) and –28% (–51 to +5), respectively. In contrast, despite a decrease in total CD8+ T cells, CD8+ CD38+ double-positive T cells increased from baseline to week 12: +28% (–18 to +242). However, CD8+ CD45RA+ T cells (which include naive and antigen-experienced CD8+ cells)20,21 showed a high but transient increase, but comparable levels were observed from baseline to week 12: +9% (–36 to +93). The kinetics of these T cell subset counts are shown in Figure 1(b).

In order to explain these immunological variations, we studied concomitantly the variations in apoptosis of CD4+ and CD8+ T cells, and of mononuclear cell proliferation, as explained in the Methods section. As shown in Figure 2(a), triple staining using CD3-FITC and CD4-PE mAbs plus Hoechst dye allowed us to quantify PCD on both T cell subsets. The percentage of spontaneous apoptotic CD4+ T cells increased from 20.5% (8–43) at baseline to 35% (9–40) at week 12 (P = 0.16). The percentage of spontaneous apoptotic CD8+ T cells increased significantly from 14.5% (9–37) to 31% (15–46) (P = 0.01). The kinetics of spontaneous apoptosis, as well as Fas-induced apoptosis of both CD3+ cell subsets and naive CD4+ T cells, are shown in Figure 2(b).

Fas stimulation with agonistic antibodies up-regulated spontaneous apoptosis at all time points, but without statistical significance whatever the T cell subset considered. During the study period, no significant relative increase in Fas-induced apoptosis compared to baseline was observed, in contrast to the increase in spontaneous apoptosis, which appeared early after STI (Figure 2b).

Our mononuclear cell proliferation assay provided evidence of significant variations in spontaneous proliferation capacities of mononuclear cells (Figure 2c). Both spontaneous and phytohaemagglutinin (PHA)-induced proliferation was increased compared with baseline, but not in similar proportions. The spontaneous proliferation index increased from 0.011 (0.01–0.04) at baseline to 0.04 (0.25–0.836) at week 12 (P = 0.01). However, spontaneous proliferation appeared to occur early and only for a short time (Figure 2c). For PHA-induced proliferation, values were 0.15 (0.001–0.423) and 0.103 (0.25–0.800), respectively (P = 0.34).

Genotypical resistance and impact on T cell apoptosis

As shown in Table 2, seven patients out of 10 exhibited no persistent primary mutation in the reverse transcriptase gene, as well as in the protease gene over 8 weeks. We then compared the impact of resistance mutation disappearance on CD4+ T cell apoptosis. As shown in Figure 3, patients with no persistent resistance mutation exhibited an increasing level of CD4+ T cell apoptosis from baseline to week 12, but without reaching statistical significance. In contrast, variations in CD4+ T cell apoptosis in patients without complete reverse resistant mutations showed a similar level of T cell apoptosis. Finally, whatever the time-point considered, the viral load did not appear to be correlated with the level of T cell subset apoptosis (data not shown).

Discussion

Our study confirms that STI leads to a rapid and important diminution of CD4+ T cells (–45%), associated with an increase in plasma viral load (+34%) and a slight decrease in CD8+ T cells (–11%). Most studies published in this field have shown similar results,1,3,5,8 even if the intensity of T cell changes may vary, depending on the viro-immunological status before STI. Our own study population, recruited
Figure 2. Up-regulation of T cell apoptosis and mononuclear cell proliferation capacities in HIV-infected patients benefiting from structured treatment interruptions. (a) Representative data from one subject: PMBC were cultured overnight and then stained with CD3-FITC and CD4-PE monoclonal antibodies, and labelled with Hoechst 33342, as described in the Methods section. For both quadrants, the percentage of positive T cells is given. The negative impact of STI was obvious on the percentage of CD4+ T cells, from 28% at baseline to 15% at week 8. Computer-assisted DNA histograms from corresponding gates indicated the percentage of apoptotic cells characterized by a distinct cell-cycle region of hypodiploid DNA peak identified below the G0/G1 diploid peak. Accordingly, the percentage of CD4 T cell apoptosis was clearly up-regulated in the same period, from 14% to 29%. In contrast, in spite of increased CD8+ T cell (i.e., CD3+ CD4– cells in this figure) apoptosis, the CD8+ T cell percentage was not dramatically modified. (b) The kinetics of spontaneous and Fas-induced apoptosis of both CD4+ and CD8+ T cells. The apoptosis of T cell subsets was measured using triple staining, as indicated in the Methods section. The kinetics of spontaneous CD4+ T cell apoptosis showed a biphasic increase, whereas CD8+ T cell spontaneous apoptosis increased in a linear fashion. No significant up-regulation of Fas-induced apoptosis could be detected for either T cell subset at week 12 compared to baseline. (c) The kinetics of spontaneous and PHA-induced proliferation capacities of PMBC over 12 weeks of STI. Proliferation was measured using a colorimetric technique and tested in triplicate, as indicated in the Method section. Both spontaneous and PHA-induced proliferation capacities appeared to be highly increased by STI compared with baseline, but only for a transient period. Mean levels ± s.e.m. are shown. A significant difference, compared with baseline value, according to Wilcoxon’s rank test is indicated as: **P < 0.01.
Apoptosis and proliferation after structured therapeutic interruption

<table>
<thead>
<tr>
<th>Patient</th>
<th>Week (W)</th>
<th>Reverse-transcriptase gene mutations</th>
<th>Protease gene mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>W0</td>
<td>67,69,70,103,184,219</td>
<td>48,54,63,71,73,77,82,90</td>
</tr>
<tr>
<td>W8</td>
<td></td>
<td>67,69,219</td>
<td>63,77</td>
</tr>
<tr>
<td>2</td>
<td>W0</td>
<td>41,184,210,215</td>
<td>36,63,71,73,90</td>
</tr>
<tr>
<td>W8</td>
<td></td>
<td>–</td>
<td>63,71</td>
</tr>
<tr>
<td>3</td>
<td>W0</td>
<td>67,70,151,184,190,219</td>
<td>10,33,63,71,73,90</td>
</tr>
<tr>
<td>W8</td>
<td></td>
<td>77</td>
<td>63</td>
</tr>
<tr>
<td>4</td>
<td>W0</td>
<td>41,67,74,106,184,190,215</td>
<td>10,36,63,71,90</td>
</tr>
<tr>
<td>W8</td>
<td></td>
<td>41,67,208,210,215</td>
<td>20,36,46,63,84,90</td>
</tr>
<tr>
<td>5</td>
<td>W0</td>
<td>67,70,106,184,219</td>
<td>10,63,90</td>
</tr>
<tr>
<td>W8</td>
<td></td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>6</td>
<td>W0</td>
<td>62,75,77,151,184</td>
<td>10,36,46,82</td>
</tr>
<tr>
<td>W8</td>
<td></td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>7</td>
<td>W0</td>
<td>41,67,69,70,75,184,190,215,219</td>
<td>10,63,71,73,77,90</td>
</tr>
<tr>
<td>W8</td>
<td></td>
<td>69,67</td>
<td>63</td>
</tr>
<tr>
<td>8</td>
<td>W0</td>
<td>41,67,70,75,101,108,184,208,210,215,219</td>
<td>10,20,36,48,54,64,71,82,84</td>
</tr>
<tr>
<td>W8</td>
<td></td>
<td>–</td>
<td>64</td>
</tr>
<tr>
<td>9</td>
<td>W0</td>
<td>62,65,70,75,77,116,151,184</td>
<td>10,20,36,48,54,63,82</td>
</tr>
<tr>
<td>W8</td>
<td></td>
<td>116</td>
<td>63</td>
</tr>
<tr>
<td>10</td>
<td>W0</td>
<td>41,67,103,181,208,210,215</td>
<td>10,36,63</td>
</tr>
<tr>
<td>W8</td>
<td></td>
<td>41,67,208,210,215</td>
<td>36,63</td>
</tr>
</tbody>
</table>

Table 2. Resistance mutation to antiretroviral compounds from baseline to week 8 in 10 HIV-1-infected patients who benefited from STI following multiple treatment failures. All mutations detected at the initiation of STI in both the reverse-transcriptase gene and protease gene, are shown here. Seven out of 10 patients (2, 3, 5–9) showed no evidence of persistent significant resistance mutations at week 8.

Among patients presenting with several virological failures, is heterogeneous (see Table 1). We show here that both naive and memory CD4+ T cells are depleted during STI. In contrast, the CD8+ CD38+ T cell subset increased early after STI, as did CD8+ CD45RA+ T cells. Accordingly, previous studies have shown that CD8+ CD38+ T cells include truly naive cells and antigen-experienced cells, which can be distinguished by the membrane expression of CD27 and CCR7. The increase in CD8+ T cells is discussed below.

A recent report indicated that the decrease in CD4+ T cell count occurring in STI was related to the re-emergence of drug-susceptible wild-type virus, suggesting that these immunological alterations may be caused by the increase in viral fitness. However, direct HIV cellular toxicity still needs to be demonstrated fully, and indirect mechanisms have been put forward to explain CD4+ T cell lymphopenia. Since STI appears to induce a period of intense immunological changes, we considered measuring apoptosis in T cell subsets and proliferation modifications.

Our results indicate that STI is associated with both an up-regulation of apoptosis in T cell subsets, and an early and transient increase in proliferation of PBMC. Spontaneous CD4+ T cell apoptosis appears to be up-regulated earlier than CD8+ T cell apoptosis, but is also biphasic with a decrease between weeks 2 and 6 and a second rise thereafter (see Figure 2b). This pattern of CD4+ T cell apoptosis is similar to that of both naive and memory CD4+ T cell counts. In contrast, the augmentation in CD8+ T cell apoptosis was linear over 12 weeks and did not fit with variations in CD8+ T cell counts. In addition, we did not observe significant variations in Fas-induced apoptosis, whatever the T cell subset considered.

Proliferation of mononuclear cells appears to rise early during STI, but only for a short period. This result is in accordance with previous studies, and is thought to represent endogenous immunization as a result of active viral replication. Indeed, we observed an early increase in CD8+ CD38+ cells and CD8+ CD45RA+ T cells (Figure 1b), which is in accordance with previous studies showing that proliferation of T cells in HIV-infected patients concerned mainly CD8+ T cells. Moreover, studies showed that STI leads to expansion of differentiated CD8+ T cells when viral rebound occurred, and that in contrast, the increase in HIV-specific CD4+ T cells was only transient and mainly observed in patients with low viral load at baseline. This expansion of CD8+ T cells may explain the increase in proliferation of mononuclear cells observed in our study (see Figure 2c). It should be noted that this putative specific immunological activation was unable to control HIV replication, as demonstrated by the viral load curve (see Figure 1a). Furthermore, this cellular proliferation was also unable to maintain the absolute number of T cells, highlighting the role of apoptosis in the occurrence of lymphopenia.

The existence of both apoptosis and proliferation of immune cells during STI is not surprising. It is noteworthy that these cellular events have been demonstrated during primary HIV syndrome, and that STI may lead to acute retroviral syndrome. However, the precise mechanisms of up-regulation of apoptosis during STI are unknown. Spontaneous apoptosis of T cells is independent of HIV load in patients receiving antiretroviral treatment; this observation is confirmed in the present study (data not shown). In addition, CD8+ T cells mediating antibody-dependent cellular cytotoxicity (ADCC) were shown to be deleterious in both experimental and clinical studies. Thus, because STI leads to viral replication, it might be associated with increased production of viral proteins known to stimulate ADCC, such as gp120, which in turn leads to up-regulation.
of T cell apoptosis. Accordingly, CD4 stimulation by agonistic antibodies induces a higher level of CD4+ T cell apoptosis in patients with detectable viral load despite antiretroviral combinations, compared with patients with undetectable viral load.28,29,31 Moreover, reverse resistance mutations to antiviral drugs is associated with a higher viral fitness, but also with apoptosis up-regulation, as suggested by our results (see Figure 3). Further studies are needed to confirm these hypotheses.

The importance of a decrease in CD4+ T cells occurring with STI strongly suggests that STI should be limited to patients without immune defects. It is noteworthy that Deeks et al.8 reported three patients with severe adverse effects as a result of STI. Garcia et al.32 have shown that in patients with a normal immunological status at baseline, STI may lead to prolonged lymphopenia, persisting even after the re-introduction of antiretroviral treatment.32 In the same way, reports indicate that STI was not followed systematically by significant up-regulation of HIV-specific clones, but if so, only for a short period.36,32,33 As an explanation for these results, our present study suggests strongly that during STI apoptosis of T cells is an overwhelming phenomenon compared to T cell proliferation. Accordingly, immunological benefits of STI should be only transient, and this therapeutic option considered with caution in immunodepressed patients.

References

Apoptosis and proliferation after structured therapeutic interruption


