The impact of highly active antiretroviral therapy by the oral route on the CD8 subset in monkeys infected chronically with SHIV 89.6P

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Abstract

The objective of this study was to assess the impact of highly active antiretroviral therapy (HAART) by an oral route on the peripheral blood CD8 subset in the monkeys infected persistently with a pathogenic strain, SHIV 89.6P. Two rhesus macaques were inoculated intravenously with SHIV 89.6P, then treated with the combination of AZT, 3TC and Lopinavir/Ritonavir (LPV/RTV) as recommended in humans by the oral route with confectionery continued for 28 days. In one of two chronically infected macaques, MM260, the viral load was maintained in the range of 10^4 – 10^5 copies/ml before HAART. The plasma viral load and proviral DNA decreased dramatically during the treatment, and cessation of this therapy the viral load rebounded to the pre-treatment level but the proviral DNA rebound was delayed. The other monkey, MM242, had low viral loads (1.2 x 10^3 – < 5 x 10^2 copies/ml) both before and after HAART. CD4^+ and CD8^+ T cell counts and proviral DNA level were not significantly changed after the treatment. The percentages of CD8^+CD45RA^-Ki67^+ cells increased during (MM260) or after (MM242) HAART and the subset was maintained at a high percentage until 18 weeks post HAART in MM242. These findings suggest that this primate model might serve an important role in testing the virological and immunological efficacy of novel therapeutic strategies combined with HAART.

Keywords: SHIV 89.6P; Antiretroviral therapy; Ki67^+; Memory CD8^+ T cells; Proviral DNA; Animal model

1. Introduction

Pre-clinical approaches in non-human primate models of AIDS enable pertinent evaluations to be carried out and the possibility to determine precisely the conditions of efficacy can be determined (Le Grand et al., 1994). Macaques infected with pathogenic strains of the simian immunodeficiency virus (SIV) or related chimeras expressing the envelope of HIV-1 (simian/human immunodeficiency virus, SHIV) are currently relevant models of human HIV infection and AIDS (Haigwood, 1999; Nath et al., 2000; Nathanson et al., 1999; Tang et al., 2002). SIV and SHIV have biological properties similar to those of HIV, and infection of macaques with pathogenic isolates induces an immunodeficiency syndrome strikingly mimicking human AIDS (Reimann et al., 1996a,b). Animal models are also useful for understanding the complexity of the pathogenic mechanisms of HIV infection during antiviral treatment (Endo et al., 2000; Enose et al., 2002; Igarashi et al., 2001).

SHIVs contain HIV-1-derived segments encoding viral envelope glycoproteins and regulatory proteins such as Tat, Rev and Vpu in the SIV background (Li et al., 1992). SHIV containing the envelope glycoproteins of a primary HIV-1 isolate, 89.6, replicated in rhesus monkeys but did not deplete CD4^+ T lymphocytes or induce disease in these animals. Serial transfer
of blood from SHIV<sub>89.6</sub>-infected monkeys to naive monkeys generated a virus, termed SHIV<sub>89.6p</sub>, that exhibited only a modest increase in replication in infected monkeys compared with SHIV<sub>89.6</sub>. However, SHIV<sub>89.6p</sub> caused rapid loss of CD4<sup>+</sup> T lymphocytes and, subsequently, AIDS-like illness in inoculated monkeys (Reimann et al., 1996a,b). An animal system using SHIV<sub>89.6p</sub> acutely infected macaques with highly active antiretroviral therapy (HAART) was the same regimen used for humans (AZT+3TC+IDV) by the oral route (Le Grand et al., 2002; Thiebot et al., 2001) and a nasogastric catheter was placed for administering the drugs (Thiebot et al., 2001).

The objective of this study was to set up a novel AIDS model in monkeys and to evaluate the efficacy of the combination of AZT, 3TC and Lopinavir/Ritonavir (LPV/RTV), when administered by the oral route to the monkeys infected chronically with the pathogenic SHIV<sub>89.6p</sub>.

2. Materials and methods

2.1. Cells and viruses

M8166 cells (Clapham et al., 1987) were grown in an RPMI-1640-based culture medium supplemented with 15% fetal calf serum (FCS: HyClone Laboratories, Logan, UT), 50 U/ml of penicillin and 50 mg/ml of streptomycin. SHIV<sub>89.6p</sub> (Reimann et al., 1996a) and HIV-1<sub>LAI</sub> (Clavel et al., 1986) were used for the drug susceptibility assay.

2.2. Antiviral agents

Zidovudine (AZT) was purchased from Sigma (St. Louis, MO), Lamivudine (3TC) was a kind gift from R.F. Schinazi (Atlanta, GA), RTV was kindly provided by Abbott Laboratories (Abbott Park, Ill.). LPV was synthesized using published methods (Carrillo et al., 1998), Kaletra™ liquid (LPV/RTV) and Combidir tablets (AZT/3TC) were purchased from Abbott Laboratories and GlaxoSmithKline, respectively.

2.3. Drug susceptibility assay

The sensitivities of SHIV<sub>89.6p</sub> against various drugs were determined as described previously with minor modifications (Maeda et al., 2001; Yoshimura et al., 1999). Briefly, M8166 cells (5 x 10<sup>5</sup> per ml) were exposed to 100 TCID<sub>50</sub> of SHIV<sub>89.6p</sub> and HIV-1<sub>LAI</sub>, in the presence of various concentrations of drugs in 96-well microculture plates and incubated at 37 °C for 7 days. After 100 µl of the medium was removed from each well, 10 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (7.5 mg/ml) in phosphate-buffered saline (PBS) was added to each well in the plate, followed by incubation at 37 °C for 2 h. After incubation, to dissolve the formazan crystals, 100 µl of acidified isopropanol containing 4% (v/v) Triton X-100 was added to each well and the optical density was measured in a microplate reader. All assays were performed in duplicate (S.D.: < 25%).

2.4. Monkeys

Two rhesus macaques (Macaca mulatta), MM260 and MM242, were infected intravenously with 1000 and 10 TCID<sub>50</sub> (50% tissue culture infectious dose) of a pathogenic SHIV<sub>89.6p</sub>, respectively. SHIV<sub>89.6p</sub> was provided by K.A. Reimann and N.L. Letvin (Beth Israel Hospital, Boston, MA). However, because the stock SHIV<sub>89.6p</sub>, which was not the original virus used in this study might have slight loss of pathogenicity in the course of stock virus preparation, the CD4 values of the monkeys inoculated with SHIV<sub>89.6p</sub> sometimes showed a some reversal (20–50% of the mean value before inoculation) after the drastic drop up to 10% at 2–3 weeks post inoculation. The two animals were treated with a combination of AZT (5 mg/kg), 3TC (2.5 mg/kg) and LPV/RTV (12/3 mg/kg) administered by the oral route with confectionery twice a day after an intravenous inoculation of SHIV<sub>89.6p</sub>. Briefly, Combidir tablets (AZT/3TC) were ground and mixed with Kaletra™ liquid (LPV/RTV) then the drug-mixture was poured into the sweet confectionery that the monkeys are fond of. We monitored the compliance of drug delivery by observing the monkeys until they had finished eating the sweets. Treatment was initiated 38 (MM260) or 84 (MM242) weeks after the inoculation of SHIV89.6P. Briefly, Combidir tablets (AZT/3TC) were purchased from Abbott Laboratories and GlaxoSmithKline, respectively.

2.5. Measuring of proviral DNA level

Proviral DNA levels in peripheral blood mononuclear cells (PBMC) from SHIV<sub>89.6p</sub>-infected-monkey were measured using a novel hypersensitive nested PCR in the LTR region. The first PCR was carried out with primers SHIV-O-S (5'‐AGGCATCTACGAGATTGGCA-3') and SHIV-O-A (5'‐ATTGAAGAGGGCTTTAAG‐CAA-3') primers in the U3/R region. The first PCR reaction mixture consisted of 0.5 µg of the proviral DNA solution, 50 mM KCl, 10 mM Tris–HCl (pH 8.0), 2 mM MgCl<sub>2</sub>, 0.01% gelatin, 0.2 mM dNTPs, 1.5 U of EX Taq DNA polymerase (Takara Shuzo Co., LTD.), and 15 pmol of each of the first PCR primers in a total volume of 30 µl. The PCR conditions used were an
initial 3 min at 94 °C, followed by 24 cycles of 30 s at 94 °C, 30 s at 65 °C, and 1 min at 70 °C with a final 10 min extension at 72 °C. The first PCR product was subsequently diluted 1000-fold, and subjected to a real-time PCR assay for measuring U3 DNA using SHIV-I-S (5'-AGACATTGGTGGCTTATGGA-3'), SHIV-I-A (5'-AAGTTGAGCTGGATGCATTA-3') and SYBR Green PCR Master Mix (Perkin–Elmer-Applied Biosystems). For amplification and detection of PCR products we preheated the samples at 50 °C for 2 min and at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 30 s, using an ABI PRISM 7700 sequence detection system (Perkin–Elmer-Applied Biosystems). The SIVmac239 LTR sequence was cloned into pCR2.1 TOPO vector (Invitrogen), and served as a standard curve. The level of SHIV DNA was expressed as copies per micrograms of cellular DNA (copies/μg DNA). Under these conditions, the detection limit was 300 copies/μg DNA.

2.6. Detection of plasma viral RNA

Plasma viral loads were determined using Taqman RT-PCR kits (Perkin–Elmer, New Jersey, USA) and ABI Prism 7700 ( Ui et al., 1999 ). Absolute copy numbers of viral RNAs were determined using standard plasma samples, the copy numbers, of which were determined by Chiron Corporation. Under these conditions, the detection limit was 500 copies/ml.

2.7. FACS analysis

Whole blood samples of the monkeys were stained with fluorescently labeled mouse monoclonal antibodies as follows; phycoerythrin (PE)-conjugated anti-human CD4 (NU-TiH/N, Nichirei, Japan), PerCP-conjugated anti-human CD8 monoclonal antibody (Leu-2a, BD Pharmingen, CA). After hemolysis of the blood using a lysis solution (BD Pharmingen), the, respectively, labeled lymphocytes were analyzed using FACSCalibur (BD Pharmingen).

PBMCs were also isolated from the macaques using centrifugation on Ficoll–Hypaque density gradient centrifugation. The PBMCs were incubated with fluorochrome-labeled specific monoclonal antibodies against surface antigens, allophycocyanin (APC)-conjugated anti-human CD8 (RPA-T8, BD Pharmingen) and PE-conjugated anti-human CD45RA (SH9, BD Pharmingen), and also incubated with 7-amino-actinomycin D (7-AAD) (BD Pharmingen) to exclude dead cells. After fixation and permeabilization, the cells were incubated with Ki67-FITC (B56, BD Pharmingen). The stained cells were analyzed by four-color flow cytometry using a FACSCalibur (Kimura et al., 2002).

2.8. Statistical analysis

The CD4+ and CD8+ T cell counts between pre-HAART and baseline or post-HAART were determined statistically using Student’s t-test.

3. Results

3.1. In vitro drug sensitivity of SHIV89.6P and HIV-1LA1

We first tested two nucleoside reverse transcriptase inhibitors (NRTIs), AZT and 3TC, and two protease inhibitors (PIs), RTV and LPV, against SHIV89.6P and HIV-1LA1 in M8166 cells (Table 1). Antiviral activity of the two NRTIs was comparable against these two viruses. On the other hand, as shown in Table 1, SHIV89.6P had a high level of resistance to RTV (15–18-fold increase in IC50) and a moderate level of resistance to LPV (5–6-fold) compared with the case against HIV-1LA1. This finding is similar to a pattern of sensitivity of HIV-2 strains to PIs as reported (Yoshimura et al., 1999).

3.2. SHIV89.6P-infected macaques were treated with HAART by the oral route

The replication of SHIV89.6P was inhibited by AZT, 3TC or LPV within the IC50 range between 0.007 and 0.47 μM in vitro (Table 1). We selected these three drugs for treatment to monkeys infected with SHIV89.6P, because this combination is currently recommended for treatment of patients with HIV-1 infection. Two rhesus macaques were inoculated intravenously with 1000 and 10 TCID50 of a cell-free stock of SHIV89.6P for MM260 and MM242, respectively. The monkeys, MM260 and MM242, became infected and were treated with HAART initiated at 38 and 64 weeks and continued until at 42 and 68 weeks post-inoculation, respectively. The animals were treated, as recommended in the case of humans, by the combination of AZT (5 mg/kg bid), 3TC (2.5 mg/kg bid) and LPV/RTV (12/3 mg/kg bid) after the intravenous inoculation of SHIV89.6P. This treatment was administered by the oral route together with confectionery and was continued for 28 days. The animals (MM260 and MM242) were monitored for CD4+ and CD8+ T cell counts, viral loads in plasma, proviral DNA and CD8+ T cell turnover.

MM260, the monkey with the high viral load showed a significant decline in CD4+ but not CD8+ T cell counts before HAART compared with the pre-infection level (598 ± 80 vs. 49 ± 16 per μl in mean CD4+ count ± S.D., P < 0.001 determined by the Student’s t-test, 288 ± 46 vs. 221 ± 81 per μl in mean CD8+ count ± S.D., P = 0.25). CD4+ and CD8+ T cell counts

increased during and after treatment compared with the pre-HAART level (49 ± 16 vs. 99 ± 26 per µl in mean CD4⁺ count ± S.D., \( P = 0.002 \), 221 ± 81 vs. 392 ± 108 per µl in mean CD8⁺ count ± S.D., \( P = 0.01 \)) (Fig. 1A). At week 38, when HAART was initiated, the plasma viral load in MM260 was 3.4 × 10⁴ copies/ml, and the proviral DNA was 6.8 × 10⁴ copies/µg DNA. After commencement of HAART, the viral RNA in the plasma declined to below the threshold of detection within 3 weeks post HAART. On the other hand, proviral DNA remained detectable under the treatment though a continuous decline in the level was observed at 9 weeks post HAART (Fig. 1B).

In the low viral load monkey, MM242, the CD4⁺ cell numbers were maintained over 400 per µl before HAART but the macaque also showed a decline in CD4⁺ but not CD8⁺ T cell counts before HAART compared with the pre-infection level (2152 ± 542 vs.

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**Table 1**

Sensitivities of SHIV₈₉₋₆P and HIV-1LAI to RTIs (AZT and 3TC) and PIs (LPV and RTV)

<table>
<thead>
<tr>
<th>Virus</th>
<th>Cells</th>
<th>IC₅₀ (µM) ± S.D.⁻ᵃ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>AZT</td>
</tr>
<tr>
<td>SHIV₈₉₋₆P</td>
<td>M8166</td>
<td>0.007 ± 0.002</td>
</tr>
<tr>
<td>HIV-1LAI</td>
<td>M8166</td>
<td>0.013 ± 0.001</td>
</tr>
</tbody>
</table>

⁻ᵃ Data shown represent mean values (with standard deviations) derived from the result of two independent experiments conducted in duplicate.

RTIs, reverse transcriptase inhibitors; AZT, zidovudine; 3TC, lamivudine. PIs, protease inhibitors; LPV, lopinavir; RTV, ritonavir.

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Fig. 1. Analyses of SHIV₈₉₋₆P-infected rhesus macaque MM260 treated with LPV/r, AZT and 3TC for 4 weeks by the oral route. CD4⁺ and CD8⁺ T cell counts (A), and plasma viral load and proviral DNA copy number (B) assessed within the peripheral blood are shown. The treatment period is shaded.
577 ± 139 per μl in mean CD4+ count ± S.D., \( P < 0.001, 761 ± 248 \) vs. \( 447 ± 188 \) per μl in mean CD8+ count ± S.D., \( P = 0.09 \). However, there was no significant difference between pre- and after HAART in CD4+ and CD8+ T cell counts (577 ± 139 vs. 569 ± 180 per μl in mean CD4+ count ± S.D., \( P = 0.93, 447 ± 188 \) vs. \( 385 ± 159 \) per μl in mean CD8+ count ± S.D., \( P = 0.52 \) (Fig. 2A). The plasma viral load in MM242 was detectable at one point (9 weeks before HAART) before the treatment, and was never detected during and after treatment. The proviral DNAs in MM242 were also low (<300–1590 copies/μg DNA), and was maintained below the threshold of detection after the discontinuation of HAART (Fig. 2B).

3.3. Analysis of CD8+Ki67+ T cells in SHIV89.6P-infected macaques with HAART

We determined Ki67-positive CD8+ T cells in SHIV89.6P-infected and uninfected monkeys using four-color flow cytometry analysis (Sachsenberg et al., 1998; Sodora et al., 2002). In the SHIV89.6P-infected animal with a high viral load, MM260, growth fraction (percentage of Ki67) of CD8+ T cells (10.98%) before HAART was higher than the value in the uninfected monkey (1.07%). The Ki67-positive CD8+ T cells in MM260 were increasing during HAART, especially in the CD45RA− (CD8 memory) subset. After the discontinuation of HAART, the percentage of CD8+Ki67+ subset declined to the pre-treatment level (Fig. 3A and B). In MM242, the percentage of CD8+Ki67+ subset at 4 weeks before HAART was comparable to that of the uninfected monkey (0.83 vs. 0.85%). Ki67 levels were elevated after the treatment and were maintained at a high level until 18 weeks post HAART (7.95%). CD8+CD45RA+Ki67+ T cells in MM242 increased after HAART, however, the CD8+CD45RA−Ki67+ T cells were also increasing and overcame the percentage of CD8+CD45RA+Ki67+ T cells by 13 weeks post HAART (Fig. 3C and D). We also determined Ki67 positivity in CD8+CD56+ T cells in MM242 at pre- and post-HAART. The percentages of CD56+Ki67+ were 0% and 0.05% in the CD8+ T cell population at pre- (−3 weeks) and post- (22 weeks) HAART, respectively, using flow cytometry analysis. There

Fig. 2. Analyses of SHIV89.6P-infected rhesus macaque MM242 treated with LPV/r, AZT and 3TC for 4 weeks by the oral route. CD4+ and CD8+ T cell counts (A), and plasma viral load and proviral DNA copy number (B) assessed within the peripheral blood are shown. The treatment period is shaded.
seemed to be little contribution of CD8⁺/NK cells in our data on CD8⁺Ki67⁺T cells.

4. Discussion

This is apparently the first demonstration that the model of chronically SHIV89.6P infected-monkey with a therapeutic design recommended for humans by the oral route is feasible. The reduction of viral load and proviral DNA during the chronically infected stage was observed in treated animals together with the increase of CD4 and CD8 positive T lymphocyte subsets, especially memory T cells. Moreover, in both macaques, the percentages of CD8⁺Ki67⁺ cells increased during HAART, especially in the low viral load monkey (MM242) and the subset was maintained at a high percentage until 18 weeks post HAART. In CD4 positive T cells, Ki67⁺ cells were also increased until 18 weeks post HAART in MM242 (data not shown). These results suggest that the oral treatment system in chronically infected-monkeys may prove to be a useful tool for monitoring immunological changes undergoing novel antiviral strategies with the usual anti-HIV treatment regimen.

The recent use of HAART has indicated that the decline of viral load in plasma and tissues generally results in an increase of T cell counts in peripheral blood (Autran et al., 1997; Evans et al., 1998; Mezzaroma et al., 1999; Pakker et al., 1998), which is often associated with improvement in clinical and immunological status (Autran et al., 1997; Evans et al., 1998). The mechanism(s) involved in such a peripheral T cell repopulation is unclear and may reflect the contribution of T cells from lymphoid tissues as a consequence of the reduced antigenic stimulation (Autran et al., 1997; Pakker et al., 1998), the capability for de novo production of naïve T cells (Hellerstein et al., 1999), and a decreased rate of T cell loss, which may reflect a reduced T cell susceptibility to apoptotic stimuli (Gougeon et al., 1999).

The issue of T lymphocyte turnover is central to understanding of HIV-1 pathogenesis (Antia and Halloran, 1996). Sachsenberg et al. estimated that HIV-1-infected individuals had a mean 6-fold increase in CD8⁺ T cell turnover, whereas the mean turnover of CD4⁺ T cells only increased by 2-fold (Sachsenberg et
The higher turnover of CD8+ T cells reflects the inversion of the CD4+/CD8+ ratio in HIV-1-infected patients (Chun et al., 2002; Margolick et al., 1995). In those studies, differences in CD4+ and CD8+ T cell turnover also depend on stage of the disease, as defined by CD4+ T cell counts.

CD8+ T cell counts and the percentage of CD8+CD45RA− (memory) T cells in both chronically infected macaques increased after oral route treatment. These findings were usually seen in clinical cases at the beginning of HAART (Autran et al., 1997; Badley et al., 1999). These memory cells are enriched in cells of the CD28+/CD27+ phenotype that proliferate but do not have reduced CTL activity (Appay et al., 2002). Whether or not the proliferating CD8+ T cells in our macaques given HAART are cells of this phenotype will need to be determined.

The plasma HIV-1 RNA is widely considered to be a direct indicator of the overall level of HIV-1 expression in infected individuals. However, it was reported that the concentration of HIV-1 DNA in PBMC complements the HIV-1 RNA load in plasma in predicting the clinical outcome of HIV-1 disease (Kostrikis et al., 2002). In our findings, especially in MM242 with a low plasma SHIV89.6P RNA load, the proviral DNA also may be an indicator of residual replication when plasma RNA loads were undetectable as seen in HIV-1 infected patients (Kostrikis et al., 2002; O'Doherty et al., 2002).

These monkey model given antiviral drugs by the oral route will enable the investigation of immunological changes during novel drug testing and also antiviral strategies combined with HAART.

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