A possible origin of emerged HIV-1 after interrupting anti-retroviral therapy

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ABSTRACT

Although HIV-1 can be successfully eradicated from the circulating blood of HIV-1-infected individuals using anti-retroviral therapy (ART), HIV-1 virions emerge immediately after the interruption of ART. This study was aimed to investigate the origin of the emerged HIV-1. After obtaining informed consent, blood samples from nine HIV-1-infected individuals and endoscopic ileum samples from five of the individuals were obtained. Purified peripheral mononuclear cells (PBMCs) and ileum cells were analyzed by flow-cytometry, and the V3 loop sequences of the HIV-1 envelope protein were determined. By comparing the V3 loop sequences of the samples, we confirmed that the provirus hidden in the CD4⁺ PBMCs was not the source of the HIV-1 that emerged after the interruption of ART. Although free virus and HIV-1-p24 antigen (p24)-positive cells were not seen in the blood of patients receiving ART, proviral DNA and p24 could be detected in the ileum from the same patient. Among the HIV-1-infected CD4⁺ cells in the ileum samples, Vα24⁺ natural killer T (NKT) cells were the major p24-positive cells. These results suggest that the innate NKT cells in the mucosal compartment are the most likely candidates for the origin of the HIV-1 that emerged after ART was interrupted.

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ervoir that contains the HIV-1-infected cells during ART urgently needs to be identified.

Two distinct types of chemokine receptors that serve as viral entry receptors for HIV-1 infection have been identified: CXCR4 for T cell-tropic HIV-1 (X4) and CCR5 for macrophage-tropic HIV-1 (R5) (1). R5-tropic HIV-1 is usually found early in the course of infection and is observed in the circulating blood and the mucosal compartment. X4-tropic HIV-1 is most often observed in patients who have advanced to AIDS (15). ART is widely used for the treatment of HIV-1, and R5-tropic HIV-1 has become the most prevalent strain. CXCR4 is predominantly expressed on resting, naïve T-cell subsets of the CD4+ T cells observed in the circulating blood, whereas CCR5 is almost exclusively expressed on the activated or memory T-cell subsets (8). Therefore, only primed, memory CD4+ T cells are susceptible to R5-tropic HIV-1 strains. Also, CD4+ type-I NKT cells bearing an invariant pair of T-cell receptors (TCRs; Vα24 and Vβ11) that can be differentiated from PBMCs by incubation with α-galactosylceramide (α-GalCer) (7) express very high levels of CCR5 and low levels of CXCR4. Indeed, it has been reported that CD4+ NKT cells are highly susceptible to R5-tropic HIV-1 strains and can produce 10–20-fold more viral particles than conventional CD4+ T cells (13).

In the present study, we aimed to identify the HIV-1-infected cells present in the blood and endoscopic ileum samples by analyzing the V3-loop sequence (10, 16) of the HIV-1 envelope protein. After confirming that the provirus hidden in the CD4+ PBMCs was not the origin of the emerged HIV-1 after interruption of ART, we found that Vα24+ NKT cells were the major HIV-1-p24 antigen (p24)-positive cells in the ileum from the same patient. Based on these observations, we would like to propose that the innate CD4+ NKT cells in the mucosal compartment served as the origin of the emerged HIV-1 after the interruption of ART.

MATERIALS AND METHODS

Patients. The nine patients listed in Table 1 were involved in the present experiments. The patients were all male. Among these patients, endoscopic biopsies were performed on five male patients (No. 1, 2, 7, 8 and 9) after obtaining informed consent. Five patients were not treated with ART (No. 1, 2, 3, 4 and 7), and four patients (No. 5, 6, 8 and 9) were treated. Although the viral loads of the ART patients were all undetectable during the treated periods, patients No. 5 and 6 had to stop ART because of the unfavorable side effect of the drugs, and new type viruses emerged after the interruption. This study was approved by the Review Board of Nippon Medical School and that all human participants gave written informed consent.

Isolation of cells from blood and the end of the ileum. After receiving informed consent, PBMCs were separated from the buffy coats of HIV-1-infected patients using Ficoll-Hypaque (Amersham-Pharmacia Biotech, Uppsala, Sweden) and were cultured in complete T-cell medium (CTM) (17) composed of RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), 5 mM HEPES buffer (Gibco BRL, Grand Island, NY), 100 U/mL penicillin (Gibco-BRL), 100 μg/mL streptomycin (Gibco-BRL), 2 mM L-glutamine (Gibco-BRL), 2 mM sodium pyruvate (Gibco-BRL), 2 mM non-essential amino acids (Gibco-BRL), 2 mM modified vitamins (Gibco-BRL), and 0.5 μM 2-mercaptoethanol (2-ME) (Gibco-BRL). Also, the ileum tissue samples were obtained by endoscopic biopsies. The cells were then shaken in RPMI-1640 media (Invitrogen Life Technologies, Carlsbad, CA) with 10% FCS (HyClone, Logan, UT) and 0.5% collagenase D (Wako Pure Chemical Industries, Osaka, Japan) for 20 min at 37°C. The collagenase-treated ileum tissue samples were then placed on a stainless mesh, mashed and washed twice with PBS to obtain the purified ileum cells containing both CD3-positive T lymphocytes and CD3-negative non-T cells such as macrophages and dendritic cells (DCs), as described previously (18).

Separation of purified ileum cells into CD3-positive and -negative cells using microbeads. The obtained ileum cells were separated into CD3-positive and -negative cells using anti-CD3 microbeads (Miltenyi Biotec, Auburn, CA) and a magnetic cell separator (MACs; Miltenyi Biotec).

DNA and RNA extraction and PCR analysis. Genomic DNA was extracted from PBMCs and the purified ileum cells using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). Free viral RNA was extracted from the serum using a High Pure Viral RNA Kit (ROCHE, Basel, Switzerland), and first-strand DNA was synthesized by RT-PCR, as described recently (11). Then, the V3-loop region was amplified by nested PCR. The primer sets were as follows: V3 loop region sense 1, 5’-AAT GTC AGC ACA GTA CAA TGT ACA C-3’; antisense 1,
Sequencing of the V3-region of the HIV envelope protein and constructing a phylogenetic tree. The plasmids were amplified by PCR using a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). The primer used for the PCR was 5′-TAA TAC GAC TCA CTA TAG GG-3′ (12). After the PCR amplification, each V3-sequence was determined using a 3130 Genetic Analyzer (Applied Biosystems) with Data Collection Software Version 3.0 (Applied Biosystems), and the sequences were analyzed with Sequencing Analysis Software v5.3.1 (Applied Biosystems). Based on the obtained sequences, a phylogenic tree was constructed using the Unweighted Pair-Group Method with Arithmetic mean (UPGMA) on the Kyoto University Bioinformatics Center server (http://www.genome.jp/tools/clustalw/).

DNA cloning. The PCR products were inserted into plasmid vectors using a TA cloning kit (Invitrogen). The plasmids were transformed into E. coli Top10 competent cells by electroporation (2.48 V). After the transformation, the competent cells were shaken for 1 h at 37°C in SOC medium composed of 20 mg/mL Bacto Tryptone, 5 mg/mL Bacto Yeast Extract, 0.5 mg/mL NaCl, 5 NaOH (0.2 mL/L), 1 M MgCl₂ (10 mL/L) and 1 M MgSO₄ (10 mL/L) and incubated overnight at 37°C in culture dishes containing Lysogeny Broth (Invitrogen; 2 g/dL) and 1.5% Select Agar (TAKARA BIOTECHNOLOGY, Shiga, Japan). Then, individual colonies were grown in Lysogeny Broth (2 g/dL) in tubes overnight with shaking at 37°C. The plasmids with individual V3-loop region were extracted using a Wizard® Plus SV Miniprep DNA Purification System (Promega, Fitchburg, WI).

<table>
<thead>
<tr>
<th>Pt</th>
<th>Age (y)</th>
<th>Duration of ART (months)</th>
<th>CD4 (μL)</th>
<th>VL (copy/mL)</th>
<th>ART</th>
<th>Endoscopic Biopsy</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>31</td>
<td>—</td>
<td>278</td>
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<td>untreated</td>
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</tr>
<tr>
<td>2</td>
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<td>—</td>
<td>44</td>
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<td>+</td>
</tr>
<tr>
<td>3</td>
<td>75</td>
<td>—</td>
<td>565</td>
<td>55000</td>
<td>untreated</td>
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</tr>
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<td>4</td>
<td>72</td>
<td>—</td>
<td>67</td>
<td>41000</td>
<td>untreated</td>
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</tr>
<tr>
<td>5</td>
<td>25</td>
<td>58</td>
<td>225</td>
<td>11000</td>
<td>interruption</td>
<td>—</td>
</tr>
<tr>
<td>6</td>
<td>51</td>
<td>22</td>
<td>286</td>
<td>6800</td>
<td>interruption</td>
<td>—</td>
</tr>
<tr>
<td>7</td>
<td>35</td>
<td>—</td>
<td>368</td>
<td>14000</td>
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<td>+</td>
</tr>
<tr>
<td>8</td>
<td>53</td>
<td>14</td>
<td>280</td>
<td>undetected</td>
<td>EPZ/LPV/r</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>49</td>
<td>16</td>
<td>245</td>
<td>undetected</td>
<td>TVD/ATV/r</td>
<td>+</td>
</tr>
</tbody>
</table>

Pt, Patient (all patients were male); ART, anti-retroviral therapy; VL, viral load; EPZ, Abacavir + Lamivudine; LPV, Lopinavir; r, Ritonavir (for boost); TVD, Tenofovir + Emtricitabine; ATV, Atazanavir.

Table 1 List of patients

Pt, Patient (all patients were male); ART, anti-retroviral therapy; VL, viral load; EPZ, Abacavir + Lamivudine; LPV, Lopinavir; r, Ritonavir (for boost); TVD, Tenofovir + Emtricitabine; ATV, Atazanavir.

Antibodies, flow cytometric analysis and cell sorting. The APC-conjugated anti-CD3 mAb and the APC-conjugated anti-CD4 mAb were purchased from Biolegend (San Diego, CA), the FITC-conjugated anti-HIV-1-p24 (p24) mAb and the PE-conjugated anti-Vα24 mAb were purchased from Beckman Coulter (Fullerton, CA), and the PE-conjugated anti-CD11c mAb was obtained from BD Biosciences. After incubation with the relevant mAbs at 4°C for 30 min, the cells were washed and re-suspended in PBS with 2% FCS and 0.01 M sodium azide (PBS-based medium) for analysis on a FACSCanto II (BD Biosciences) with FlowJo software (TreeStar, Ashland, OR). To stain intracellular p24, the cells were fixed with 2% paraformaldehyde and permeabilized with Cytofix/Cytoperm solution (BD Biosciences) at 4°C for 20 min. After washing twice with Perm/Wash buffer (BD Biosciences), the cells were incubated with an anti-human p24 mAb at 4°C for 30 min.

RESULTS

Therapeutic, immunologic and virologic profiles of the patient

Nine male patients were involved in the experiment. Endoscopic biopsies were performed on five patients...
(No. 1, 2, 7, 8 and 9) after obtaining informed consent (Table 1). Five patients were not treated with ART (No. 1, 2, 3, 4 and 7), and two patients (No. 8 and 9) were treated. The viral loads of patients treated with ART were below the level of detection. Patients 5 and 6 had previously been treated with ART but had stopped because of the side effect of the drugs. The viral load in the sera of these patients had been undetectable before the interruption of ART.

The HIV-1 that emerged after the interruption of ART was not originated from provirus hidden in PBMCs

To determine whether the HIV-1 that emerged after the interruption of ART originated from HIV-1-infected cells in the circulating blood, we first compared the third variable region of the envelope protein (V3 loop)-sequence of free HIV-1 in the sera with that of provirus DNA hidden in the PBMCs. As demonstrated in Table 2, the V3-sequence of the free HIV-1 was identical to that of the provirus in the PBMCs of each patient, when the patients were currently receiving treatment with ART (Patients No. 1, 2, 3 and 4). However, after the interruption of ART due to side effects of the drugs or other problems, the V3-sequence of the newly emerged HIV-1 in the sera was distinct from that seen in the CD4+ PBMCs of the same patient during the ART treatment, and the V3 sequences of the rebounding HIV-1 indicated that the virions were R5-type variants, according to the “geno2pheno coreceptor program” (http://corereceptor.bioinf.mpg.de/) (Table 2; Patients No. 5 and 6). These results suggest that HIV-1 hidden in the CD4+ PBMCs was not the origin of the newly emerged HIV-1 after the interruption of ART.

Table 2  Comparison of the V3-loop region sequence in the HIV-1 envelope proteins of free serum viruses with those seen in the CD4+ PBMCs of the same patient

<table>
<thead>
<tr>
<th>Pt.</th>
<th>Analyzed Materials</th>
<th>V3-loop Sequence (Residue No.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>305</td>
</tr>
<tr>
<td>1</td>
<td>PBMC provirus DNA:</td>
<td>CTRPNNTTRK SIHIGPGRAF FAT-DIIGDI RQAHCN</td>
</tr>
<tr>
<td></td>
<td>Serum virus:</td>
<td>CTRPNNTTRK SIHIGPGRAF FAT-DIIGDI RQAHCN</td>
</tr>
<tr>
<td>2</td>
<td>PBMC provirus DNA:</td>
<td>CTRPNNTTRK GIHSGPGGTI YATGAIIGDI RQAHCN</td>
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<tr>
<td></td>
<td>Serum virus:</td>
<td>CTRPNNTTRK GIHSGPGGTI YATGAIIGDI RQAHCN</td>
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<td>PBMC provirus DNA:</td>
<td>CTRPNNTTRK GIHIGPGGAF YATGDIIGDI RQAHCN</td>
</tr>
<tr>
<td></td>
<td>Serum virus:</td>
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</tr>
<tr>
<td>4</td>
<td>PBMC provirus DNA:</td>
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<td>5</td>
<td>PBMC provirus DNA:</td>
<td>CTRPNNTTRK SIHSGPGRAW YTTGQIIGDI RKAHCN</td>
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<tr>
<td></td>
<td>Serum virus:</td>
<td>CTRPNNTTRK SIHSGPGRAW YTTGQIIGDI RKAHCN</td>
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<td>6</td>
<td>PBMC provirus DNA:</td>
<td>CTRPNNTTRK SISHPGPTI YTTGDIIGDI RQAHCN</td>
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<tr>
<td></td>
<td>Serum virus:</td>
<td>CTRPNNTTRK SISHPGPTI YTTGDIIGDI RQAHCN</td>
</tr>
</tbody>
</table>

PBMCs; peripheral blood mononuclear cells. The amino acids that differ between the proviral DNA and free serum virus in the same patient are shown in bold. 'Sequence residue number was based on the numbering reported by Ratner et al. (19).

Detection and comparison of HIV-1-infected cells in the ileum and PBMCs of patients treated with ART

Because the PBMCs in the circulating blood were not the origin of rebounding HIV-1, we sought to identify the source of HIV-1 that emerged after the interruption of ART. SIV (simian immunodeficiency virus) has been shown to accumulate at increased
Patient 1. CD4: 278 /µL VL: 38000 copy/mL ART(−)

Residue No. | 305 | 315 | 325 | 335
---|---|---|---|---
PBMCD3⁺: CRTPNNNTRK SIHIGPGRAF YATDIIGDI RQAHCN a)  
PBMCD3⁻: CRTPNNNTRK SIHIGPGRAF YATDIIGDI RQAHCN b)  
IleumCD3⁺: CRTPNNNTRK SIHMGPGRAF YATDIIGDI RQAICT c)  
IleumCD3⁻: CRTPNNNTRK SIHMGPGRAF FATDIIGDI RQAICT d)  
free virus: CRTPNNNTRK SIHIGPGRAF YATDIIGDI RQAHCN e)

**Fig. 1** The phylogenetic analysis indicates that HIV-1 is compartmentalized within the patients. Ileum samples were divided into CD3-positive or negative cells using microbeads, and their V3-sequences were determined. Based on the obtained V3-sequences, a phylogenetic tree was made using the Unweighted Pair-Group Method with Arithmatic mean (UPGMA).
levels in local mucosal compartments, such as the ileum of rhesus macaques and sooty mangabeys (9). Furthermore, even though serum HIV-1 levels drop below the limit of detection while patients receive ART, HIV-1 RNA is still detected in the feces and urine of the patients (2). These findings suggest that ART is not able to eradicate HIV-1 virions from the gastro-intestinal tract of the patients.

Therefore, we performed endoscopic biopsies on patients (No. 1, 2, 7, 8 and 9) to obtain ileum samples and analyzed the samples for the presence of HIV-1-replicating cells by flow-cytometry. As demonstrated in Fig. 2, the HIV-1-p24 antigen was detected in the CD3+ cells, including CD4+ Vα24+ NKT cells, as well as CD11c+ DCs/macrophages, in both the PBMCs and the ileum samples when an HIV-1-infected patient (No. 7) was not treated with ART. Similarly, we performed endoscopic biopsies on patients receiving ART (No. 8 and 9). To our surprise, even when the viral load in the sera was below the limit of detection and the p24 antigen was not observed in the PBMCs, provirus DNA and the p24 antigen could be detected in the ileum samples from the same patient (No. 8 and 9) (Fig. 2). Moreover, it should be noted that among the HIV-1-infected cells, the majority of the p24-positive cells in the ileum of a patient treated with ART were CD4+ Vα24+ NKT cells (Fig. 2, Patient 9). These results suggested that ART was not able to eradicate HIV-positive cells completely from the ileal mucosa and indicated that innate CD4+ Vα24+ NKT cells serve as the major reservoir of R5-HIV-1 in the mucosal compartment during ART.

DISCUSSION

In the present study, we first confirmed that the V3-sequence of the free HIV-1 in the serum was identical to that of the provirus in the PBMCs of several patients that were not treated with ART. However, when the patients were treated with ART, free virus and HIV-1-p24-positive cells disappeared from the blood, but proviral DNA and p24-positive cells with a distinct V3-sequence were detected in the ileum of these same patients. These findings indicate that the free virions in the serum originate from viral genomes hidden in CD4+ PBMCs of patients as long as they are not treated with ART. Once these patients begin ART, the release of free HIV-1 from CD4+ PBMCs is halted. However, virions may be produced and released from a separate compartment when ART is interrupted. Moreover, as shown in Table 2, the viruses that emerged after interruption of ART were R5-HIV-1. These findings are compatible with those reported by Chun et al. (3) in that the rebounding plasma virus was genetically distinct from the cell-associated HIV-1 RNA and the replication-competent virus detected within the latently infected CD4+ T cells in other persistent reservoirs, such as the mucosal compartments.

We showed here that the majority of the virions that emerged after the interruption of ART were R5-HIV-1 rather than X4-HIV-1. R5-HIV-1 is usually found early in the course of infection, and X4-HIV-1 is observed most often in patients who have advanced to AIDS. After ART became widely used for the treatment of HIV-1, R5-tropic HIV-1 emerged as the most prevalent strain. This may be because ART was established to treat AIDS rather than to control the early stage of infection, and ART is more effective against X4-HIV-1 than R5-HIV-1 throughout the body and circulating blood. Consequently, R5-HIV-1 pools are maintained during ART, particularly in mucosal compartments, such as the ileum, as demonstrated in the present study. Thus, it is possible that the R5-HIV-1 emerged after ART interruption from local mucosal compartments separated from the circulating blood.

Mucosal compartments are usually separated from the blood vessels because they are the main barriers to protect against external pathogens. In the mucosal compartments, innate defense cells are arranged to prevent pathogens from entering the circulating blood. These innate defense cells include DCs, NKT cells, and γδT cells and acquired conventional T cells, such as CD4+ helper (Th) or regulatory (Treg) cells or CD8+ cytotoxic T lymphocytes (CTLs). The innate cells express predominantly CCR5, whereas the acquired cells express CXCR4. Thus, the initial targets for R5-HIV-1 are CD4+ DCs or NKT cells at the mucosal areas that can be accessed by HIV-1. CD4+ NKT cells are highly susceptible to R5-tropic HIV-1 strains and can produce 10–20-fold more viral particles than conventional CD4+ T cells (5). Therefore, we propose that the initial HIV-1 in the early stages of infection at the mucosal compartment is R5-tropic, and their initial target CD4+ DCs transmit the HIV-1 virions to CD4+ NKT cells to expand and persist.

As indicated in Fig. 2, persistent R5-HIV-1 is not strongly eliminated by ART during treatment, and virions promptly emerged when ART was interrupted. Our current observations indicate that these newly emerged R5-HIV-1 virions were released from CD4+ NKT cells in a mucosal compartment, such as the ileum. Taken together, methods to control R5-
After obtaining informed consent, we performed endoscopic biopsies on patients (No. 7, 8, and 9) to obtain ileum samples and identified p24-positive HIV-replicating infected cells by flow-cytometry. The p24-positive cells were observed in both PBMCs and ileum samples in the untreated patient (patient No. 7). Although the viral load in the blood was undetectable and the HIV-1-p24 antigen was not observed in the PBMCs in patients receiving ART, proviral DNA and the p24 antigen could be detected in the ileum samples from the same patients (patients No. 8 and 9). Acquired conventional CD4$^+$ T cells and the innate CD4$^+$ Vα24$^+$ type-I NKT cells and CD4$^+$ CD11c$^+$ DCs were identified as p24-positive and HIV-1-infected. Among the HIV-1-infected CD4$^+$ cells, the majority of the p24-positive cells were Vα24$^+$ NKT cells and DCs in the ileum of patients treated with ART.

Fig. 2 Detection and comparison of HIV-1-infected cells in the ileum and PBMCs of patients treated with ART.
HIV-1 within CD4+ NKT cells should be developed and combined with modern ART to successfully eliminate HIV-1.

Acknowledgments

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