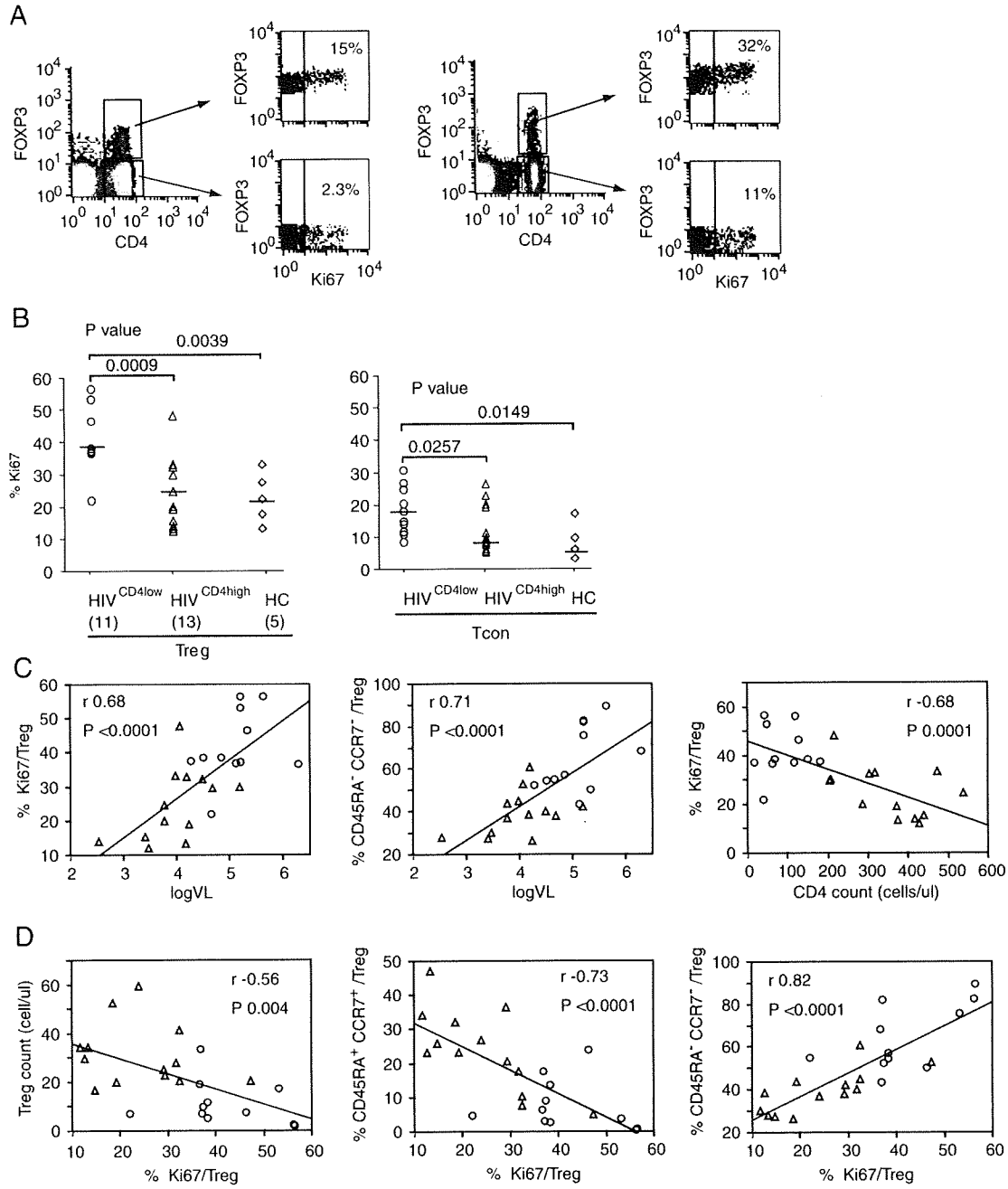

Figure 2. Ki67 staining and high proliferation rate of Treg is associated with viral load. (A) Gating of Ki67 in FOXP3$^+$ and FOXP3$^-$ CD4$^+$ cells in a healthy control (left panel) and an HIV-1-infected person (right panel). (B) Proportion of Ki67-positive Treg (left panel) is higher than that of Ki67-positive Tcon cells (right panel) in healthy controls (HC), HIV-1-infected patients with low CD4 count (HIV^{CD4low}) and HIV-1-infected patients with high CD4 count (HIV^{CD4high}) (numbers in parentheses represent the number of subjects tested). The percentages of Ki67-positive Treg and Tcon cells in the low CD4 group are higher than those in the high CD4 group and healthy control, respectively. (C) HIV-1 viral load shows a positive correlation with the percentage of Ki67 in Treg (left panel) and the proportion of CD45RA CCR7 Treg (middle panel). The CD4 count shows a negative correlation with the percentage of Ki67 in Treg (right panel). (D) The percentage of Ki67 in Treg shows correlation negatively with Treg count (left panel) and the proportion of CD45RA$^+$CCR7$^+$ Treg (middle panel) but positively with the proportion of CD45RA CCR7 Treg (right panel). Horizontal bars represent median values and p values represent results from Wilcoxon-signed rank test. Simple regression was used for correlation analysis.

PBMC and mucosal tissue [23, 32]. To investigate the impact of ART on Treg, we checked the dynamic change in Treg, their proliferation state, and subsets in nine patients until 9 months after commencement of ART (Fig. 3). The plasma viral load decreased sharply soon after commencement of ART (Fig. 3A). Associated with the decrease in viral load was a rise in the $CD4^+$ count especially in the first 2 months of ART. The CD4 count increased more than 100 cells/μL average in the first month (Fig. 3B). The absolute count of Treg increased in the first month but decreased to some extent thereafter (Fig. 3C); the frequency of Treg decreased rapidly to normal levels within 1–2 months of commencement of ART in all patients (Fig. 3D). On the other hand, the change in the proportion of Ki67 among Tcon and Treg showed a complex pattern. The proportion of Ki67 among Tcon cells increased in the first month of treatment and then decreased and fluctuated on a small scale thereafter (Fig. 3E). However, in the first 1–2 months of ART, the proportion of Ki67 among Treg decreased but maintained high levels until 9 months of ART (Fig. 3F). There was no significant change in each subset in both Treg and Tcon (Fig. 3G and H). However, the $CD45RA^-CCR7^-$ subset still accounted for a high proportion, especially in Treg (Fig. 3G and H, the right panels). The detailed change of each item in each patient is shown in Supporting Information Fig. 3. These results suggest that after initiation of ART, the slow change in the absolute number of Treg and the rapid rebound of Tcon counts resulted in a rapid normalization of the frequency of Treg in HIV-1 patients.

Discussion

Regulation of the immune response is important in maintaining self-tolerance. However, in individuals with immunodeficiency, such as patients with HIV infection, severe immune suppression may contribute to progression of AIDS. Previous studies reported activation of the immune system in HIV-1-infected patients and indicated that human $CD4^+CD25^{high}FOXP3^+$ Treg cells are derived through rapid turnover of memory populations *in vivo* [31, 33, 34].

In the present study, we found that untreated HIV-1-infected patients with low CD4 counts have a high frequency of Treg and $CD45RA^-CCR7^-$ Treg. Cell proliferation was higher in Treg than Tcon cells, especially in HIV-1 patients with low CD4 counts. In these patients, both Tcon and Treg showed a high proliferation state, particularly about 40% Treg were Ki67-positive. Ndhlovu *et al.* [22] reported that $FOXP3^+CD127^{lo}$ $CD4^+$ T cells in PBMC showed a strong negative correlation with T-cell activation during the early chronic stage of HIV infection. In our study, we also found a negative correlation between the proliferating frequency of Treg and Treg absolute count. However, we found that the proliferation of Treg correlated positively with the proportion of $CD45RA^-CCR7^-$ Treg. Furthermore, HIV viral load showed a positive correlation with both Treg proliferation and the proportion of $CD45RA^-CCR7^-$ Treg. These results suggest that HIV infection may activate Treg and result in an increased proportion of CD45RA CCR7 among Treg. On the other hand, Epple *et al.* [32] reported that the frequency and absolute counts of mucosal Treg were highly increased in untreated HIV patients. This finding may be considered another reason for our results because $CCR7^+$ lymphocytes tend to home to lymph nodes and lymphoid tissues. Therefore, we consider that in HIV-infected patients, HIV could simultaneously activate the differentiation of Treg as well as stimulate $CCR7^+$ Treg homing to lymph nodes and lymphoid tissues. These two effects of HIV on Treg result in the high frequency of Treg and a high proportion of $CD45RA^-CCR7^-$ Treg in peripheral blood in patients with low CD4 counts.

ART has been a great success in controlling HIV replication and aiding the recovery of CD4 T cells. However, data about its impact on Treg, especially in detail, are rare. In the current study, we observed that with the rapid decrease in viral load was a robust rebound of Tcon 1–2 months after ART initiation; however, the number of Treg increased in some patients but was almost unchanged in others. The unbalanced change in Tcon and Treg resulted in the frequency of Treg decreasing precipitously to normal levels in the first 1–2 months of therapy. Although the viral load decreased to a very low level in a short period after ART introduction, the proliferative state of Tcon and Treg did not decrease significantly. On the contrary, both Tcon and Treg maintained a high proliferation level, especially Treg. Moreover, the three subsets, *i.e.* $CD45RA^+CCR7^+$, $CD45RA^-CCR7^+$, and $CD45RA^-CCR7^-$ in Tcon and Treg did not show a robust change till 9 months. The results suggest that the recovery of phenotypes needs a much longer period, even if they can recover after ART.

Chase *et al.* [23] observed Treg depletion in highly active ART-treated HIV-1 patients but not in elite suppressors. Here, we did not observe depletion of Treg counts after ART introduction, but we indeed noticed a rapid normalization of the Treg frequency. As we know, to do the suppression assay *in vitro*, an appropriate ratio of Treg to responder cells is needed for observing significant suppression. Considering the suppressive function of both $CCR7^+$ and $CCR7^-$ Treg, we think that the high frequency of Treg, but not the low absolute number of Treg, provides a much better suppressive marker in treatment-naïve HIV-1 patients with low CD4 counts. On the other hand, ART may induce some improvement of the immune suppression because it could reduce the frequency of Treg.

In summary, our results of high frequencies of Treg and $CD45RA^-CCR7^-$ Treg, which tend to migrate to non-lymphoid tissues, in untreated HIV-1 patients with low CD4 counts, emphasize the potential role of Treg in immune deficiency in late-stage HIV-1 infection. Furthermore, anti-HIV treatment could result in a rapid rebound of conventional T cells but not a robust improvement of Treg within 9 months after ART initiation. The different response of Treg and Tcon to ART leads to a rapid decrease in the frequency of Treg. Recently, immune reconstitution syndrome (IRS) is becoming an important problem in HIV treatment. Most IRS occurs in 1–3 months after commencement

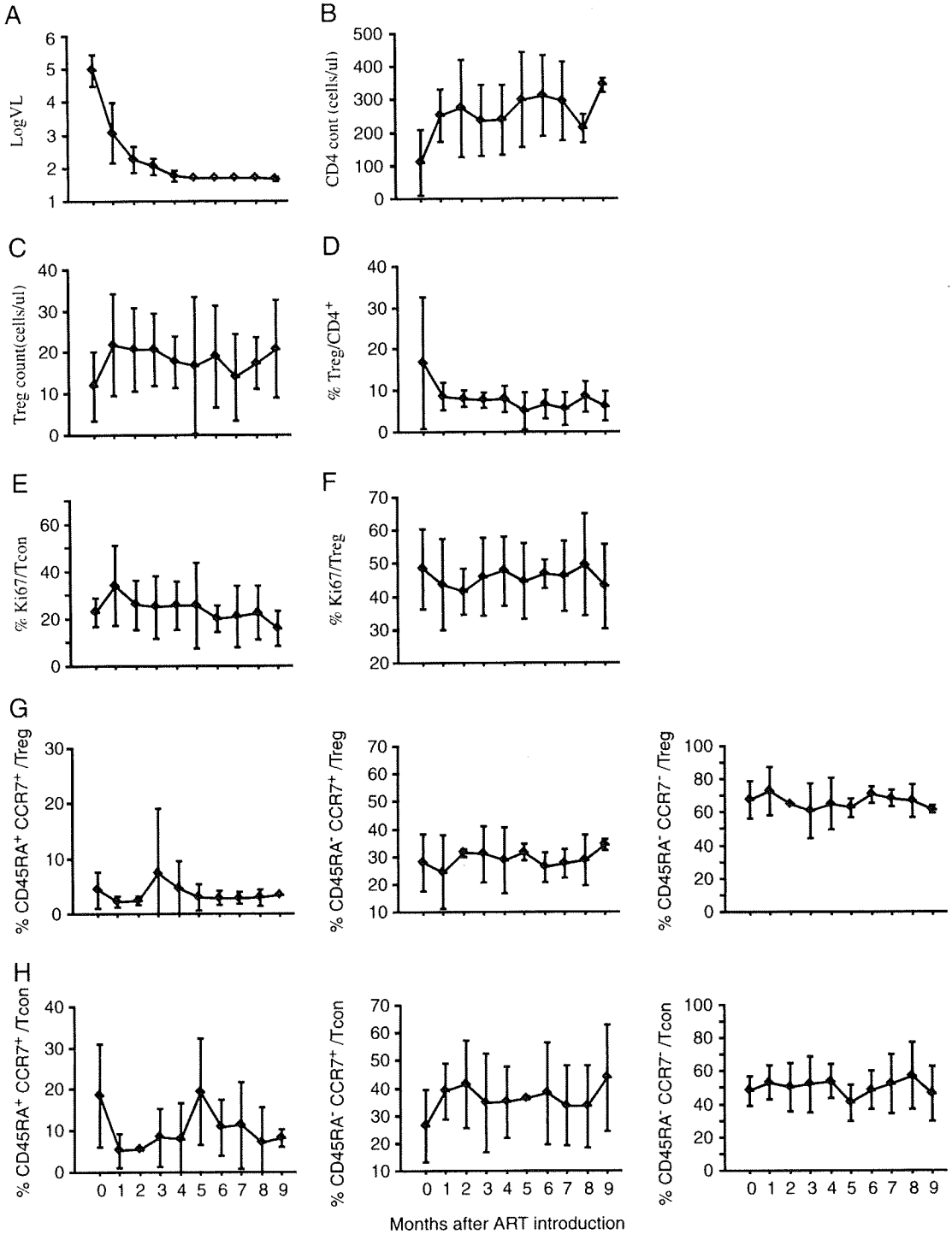

Figure 3. Serial changes in Treg and Tcon cells after commencement of ART. Commencement of ART resulted in rapid reduction in HIV viral load (A) and increase in $CD4^+$ cell count (B). Treatment caused increase in the absolute number of Treg in the first month, then fluctuated slightly thereafter (C), but resulted in a sharp decrease in their percentages in 1 month (D). The proportion of Ki67-positive Tcon increased in the first month but decreased in some extent thereafter (E), while the proportion of Ki67-positive Treg showed some change but still retained a high level at 9 months of commencement of ART (F). At 9 months after ART started, the recovery of the proportion of $CD45RA^+CCR7^+$ Treg (G, left panel) and Tcon (H, left panel) seems very slow, while the proportion of $CD45RA^-CCR7^+$ Treg (G, middle panel) and Tcon (H, middle panel) increased in some extent. However, the proportion of $CD45RA^-CCR7^-$ Treg (G, right panel) and $CD45RA^-CCR7^-$ Tcon (H, right panel) showed a small-scale change, but CD45RA CCR7 Treg maintained a high proportion till 9 months. (A–F) was from nine patients, while (G–H) was from six of them. Vertical bars represent mean ± 1SD.

of ART. Thus, we suppose that the unbalanced improvement of conventional CD4 cells and Treg after commencement of ART might be a factor for IRS. However, this issue needs more investigation.

Materials and methods

Subjects

The subjects were 95 HIV-1-infected patients who have not received any ART and gave written consent before enrollment in this study at the AIDS Clinical Center, International Medical Center of Japan, Tokyo. Nine patients who started ART were followed up for investigation of the impact of ART on Treg. Twenty-one HIV-1-negative adults were recruited as healthy controls. The demographic and clinical characteristics of the subjects are listed in Table 1. HIV-1 viral load was quantified by AMPLICOR HIV-1 MONITOR Test (Roche Diagnostics).

Cell preparation

PBMC were prepared from blood samples collected into EDTA-containing tubes by Ficoll-paque gradient centrifugation. Ki67 staining and evaluation of the ART-treated patients were carried out using cryopreserved PBMC.

For suppression assay, $CD4^+$ cells were isolated from freshly prepared PBMC by using $CD4^+$ T-cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to instructions provided by the manufacturer. $CD4^+$ cells were separated by anti-CD25 mAb (PE) and anti-PE Multisort Kit (Miltenyi) into $CD25^-$ and $CD25^+$ cells. After microbeads release, $CD25^+$ cells were sorted into $CCR7^+$ and $CCR7^-$ cells by using anti-CCR7 mAb (FITC, mouse IgG2a, R&D Systems, Minneapolis, MN) and Rat Anti-Mouse IgG2a+b Microbeads (Miltenyi). The $CD4^+CD25^-$ cells were labeled by 2 μM 5-6-CFSE as responder cells in the suppression assay. Unlabeled $CD4^+CD25^-$ cells were used as non-Treg for cell number control. PBMC that were depleted of $CD3^+$ cells by CD3 MicroBeads (Miltenyi) and irradiated with 3000 rad were used as APC.

Cell staining and flow cytometry

Freshly isolated PBMC were surface stained and also stained intracellularly for FOXP3 (PE/APC labeled, clone PCH101, eBioscience, San Diego, CA) and other markers. The stained cells were analyzed on Becton Dickinson FACSCalibur with CellQuest software (BD Bioscience, San Jose, CA). The monoclonal antibodies used in these staining procedures included anti-CCR7-FITC, anti-CD4-perCP, anti-CD25-PE, anti-CD45RA-APC/perCP, anti-Ki67-PE (BD PharMingen, San Diego, CA), and anti-CD127-FITC (eBioscience).

In vitro suppression assay

In a 96-well, round-bottom plate coated with anti-CD3 mAb (0.25–0.5 μg/mL), 5×10^4 CFSE-labeled $CD4^+CD25^-$ cells were seeded and followed by adding autologous APC (2.5×10^4). For testing Treg suppression, the same number of $CD4^+CD25^+CCR7^+$ or $CCR7^-$ cells was added as regulatory cells. In control wells, the same number of unlabeled non-Treg $CD4^+CD25^-$ cells was added in order to adjust cell numbers in each well. After 3–4 days culture in an incubator at 37°C under 5% CO_2, the cells were harvested and analyzed on FACSCalibur. Live cells were gated and the dilution of CFSE was measured as proliferation of responder cells.

Statistical analysis

Data are expressed as mean ± SD. Differences between groups or stratified groups were examined for statistical significance using Mann–Whitney *U*-test and Wilcoxon-signed rank test. Simple linear regression was used for correlation analysis. All analyses were conducted using the StatView software (version 5.0). A p value of <0.05 was considered statistically significant.

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References

1 Douek, D. C., Picker, L. J. and Koup, R. A., T cell dynamics in HIV-1 infection. *Annu. Rev. Immunol.* 2003. **21**: 265–304.

2 Gandhi, R. T. and Walker, B. D., Immunologic control of HIV-1. *Annu. Rev. Med.* 2002. **53**: 149–172.

3 Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M. and Toda, M., Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J. Immunol.* 1995. **155**: 1151–1164.

4 Mills, K. H., Regulatory T cells: friend or foe in immunity to infection? *Nat. Rev. Immunol.* 2004. **4**: 841–855.

5 Rouse, B. T., Sarangi, P. P. and Suvas, S., Regulatory T cells in virus infections. *Immunol. Rev.* 2006. **212**: 272–286.

6 Klenerman, P. and Hill, A., T cells and viral persistence: lessons from diverse infections. *Nat. Immunol.* 2005. 6: 873–879.

7 Belkaid, Y. and Rouse, B. T., Natural regulatory T cells in infectious disease. *Nat. Immunol.* 2005. 6: 353–360.

8 Coffer, P. J. and Burgering, B. M., Forkhead-box transcription factors and their role in the immune system. *Nat. Rev. Immunol.* 2004. 579: 191–198.

9 Fontenot, J. D., Rasmussen, J. P., Williams, L. M., Dooley, J. L., Farr, A. G. and Rudensky, A. Y., Regulatory T cell lineage specification by the forkhead transcription factor Foxp3. *Immunity* 2005. 22: 329–341.

10 Sakaguchi, S., Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self. *Nat. Immunol.* 2005. 6: 345–352.

11 Wu, Y., Borde, M., Heissmeyer, V., Feuerer, M., Lapan, A. D., Stroud, J. C., Bates, D. L. *et al.*, FOXP3 controls regulatory T cell function through cooperation with NFAT. *Cell* 2006. 126: 375–387.

12 Seddiki, N., Santner-Nanan, B., Tangye, S. G., Alexander, S. I., Solomon, M., Lee, S., Nanan, R. and de Saint Groth, B. F., Persistence of naive CD45RA$^+$ regulatory T cells in adult life. *Blood* 2006. 107: 2830–2838.

13 Thornton, C. A., Upham, J. W., Wikstrom, M. E., Holt, B. J., White, G. P., Sharp, M. J., Sly, P. D. and Holt, P. G., Functional maturation of CD4$^+$CD25$^+$ CTLA4$^+$CD45RA$^+$ T regulatory cells in human neonatal T cell responses to environmental antigens/allergens. *J. Immunol.* 2004. 173: 3084–3092.

14 Bacchetta, R., Passerini, L., Gambineri, E., Dai, M., Allan, S. E., Perroni, L., Dagna-Bricarelli, F. *et al.*, Defective regulatory and effector T cell functions in patients with FOXP3 mutations. *J. Clin. Invest.* 2006. 116: 1713–1722.

15 Tsunemi, S., Iwasaki, T., Imado, T., Higasa, S., Kakishita, E., Shirasaka, T. and Sano, H., Relationship of CD4$^+$CD25$^+$ regulatory T cells to immune status in HIV- infected patients. *AIDS* 2005. 19: 879–886.

16 Aandahl, E. M., Michaelsson, J., Moretto, W. J., Hecht, F. M. and Nixon, D. F., Human CD4$^+$ CD25$^+$ regulatory T cells control T-cell responses to human immunodeficiency virus and cytomegalovirus antigens. *J. Virol.* 2004. 78: 2454–2459.

17 Eggena, M. P., Barugahare, B., Jones, N., Okello, M., Mutalya, S., Kityo, C., Mugyenyi, P. and Cao, H., Depletion of regulatory T cells in HIV infection is associated with immune activation. *J. Immunol.* 2005. 174: 4407–4414.

18 Weiss, L., Donkova-Petrini, V., Caccavelli, L., Balbo, M., Carbonneil, C. and Levy, Y., Human immunodeficiency virus-driven expansion of CD4$^+$ CD25$^+$ regulatory T cells, which suppress HIV-specific CD4 T-cell responses in HIV-infected patients. *Blood* 2004. 104: 3249–3256.

19 Kinter, A. L., Hennessey, M., Bell, A., Kern, S., Lin, Y., Daucher, M., Planta, M. *et al.*, CD25(+)CD4(+) regulatory T cells from the peripheral blood of asymptomatic HIV-infected individuals regulate CD4(+) and CD8(+) HIV-specific T cell immune responses *in vitro* and are associated with favorable clinical markers of disease status. *J. Exp. Med.* 2004. 200: 331–343.

20 Andersson, J., Boasso, A., Nilsson, J., Zhang, R., Shire, N. J., Lindback, S., Shearer, G. M. and Chougnet, C. A., The prevalence of regulatory T cells in lymphoid tissue is correlated with viral load in HIV-infected patients. *J. Immunol.* 2005. 174: 3143–3147.

21 Krathwohl, M. D., Schacker, T. W. and Anderson, J. L., Abnormal presence of semimature dendritic cells that induce regulatory T cells in HIV-infected subjects. *J. Infect. Dis.* 2006. 193: 494–504.

22 Ndhlovu, L. C., Loo, C. P., Spotts, G., Nixon, D. F. and Hecht, F. M., FOXP3 expressing CD127lo CD4$^+$T cells inversely correlate with CD38$^+$CD8$^+$ T cell activation level in primary HIV-1 infection. *J. Leukoc. Biol.* 2008. 83: 254–262.

23 Chase, A. J., Yang, H., Zhang, H., Blankson, J. N. and Siliciano, R. F., Preservation of FOXP3$^+$ regulatory T cells in the peripheral blood of HIV-1-infected elite suppressors correlates with low CD4$^+$ T-cell activation. *J. Virol.* 2008. 82: 8307–8315.

24 de St Groth, B. F. and Landay, A. L., Regulatory T cells in HIV infection: pathogenic or protective participants in the immune response? *AIDS* 2008. 22: 671–683.

25 Keynan, Y., Card, C. M., Mclaren, P. J., Dawood, M. R., Kasper, K. and Fowke, K. R., The role of regulatory T cells in chronic and acute infections. *Clin. Infect. Dis.* 2008. 46: 1046–1052.

26 Sallusto, F., Lenig, D., Forster, R., Lipp, M. and Lanzavecchia, A., Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* 1999. 401: 708–712.

27 Lim, H. W., Broxmeyer, H. E. and Kim, C. H., Regulation of trafficking receptor expression in human forkhead box P3+regulatory T cells. *J. Immunol.* 2006. 177: 840–851.

28 Wei, S., Kryczek, I. and Zou, W., Regulatory T-cell compartmentalization and trafficking. *Blood* 2006. 108: 426–431.

29 Seddiki, N., Santner-Nanan, B., Martinson, J., Zaunders, J., Sasson, S., Landay, A., Solomon, M. *et al.*, Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. *J. Exp. Med.* 2006. 203: 1693–1700.

30 Liu, W., Putnam, A. L., Xu-Yu, Z., Szot, G. L., Lee, M. R., Zhu, S., Gottlieb, P. A. *et al.*, CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4$^+$Treg cells. *J. Exp. Med.* 2006. 203: 1701–1711.

31 Sousa, A. E., Carneiro, J., Meier-Schellersheim, M., Grossman, Z. and Victorino, R. M., CD4 T cell depletion is linked directly to immune activation in the pathogenesis of HIV-1 and HIV-2 but only indirectly to the viral load. *J. Immunol.* 2002. 169: 3400–3406.

32 Epple, H. J., Loddenkemper, C., Kunkel, D., Troger, H., Maul, J., Moos, V., Berg, E. *et al.*, Mucosal but not peripheral FOXP3$^+$ regulatory T cells are highly increased in untreated HIV infection and normalize after suppressive HAART. *Blood* 2006. 108: 3072–3078.

33 Hazenberg, M. D., Stuard, J. W., Otto, S. A., Borleffs, J. C., Boucher, C. A., de Boer, R. J., Miedema, F. and Hamann, D., T-cell division in human immunodeficiency virus (HIV)-1 infection is mainly due to immune activation: a longitudinal analysis in patients before and during highly active antiretroviral therapy (HAART). *Blood* 2000. 95: 249–255.

34 Vukmanovic-Stejic, M., Zhang, Y., Cook, J. E., Fletcher, J. M., McQuaid, A., Masters, J. E., Rustin, M. H. *et al.*, Human CD4$^+$ CD25^{hi} Foxp3$^+$ regulatory T cells are derived by rapid turnover of memory populations *in vivo*. *J. Clin. Invest.* 2006. 116: 2423–2433.

ART: antiretroviral therapy · **HIV-1:** HIV type 1 · **IRS:** immune reconstitution syndrome · **Tcon:** conventional CD4$^+$ T cells

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An 11-Year Surveillance of HIV Type 1 Subtypes in Nagoya, Japan

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Abstract

To monitor active HIV-1 transmission in Nagoya, Japan, we have been determining the subtypes of HIV-1 infecting therapy-naive individuals who have newly visited the Nagoya Medical Center since 1997. The subtypes were determined by phylogenetic analyses using the base sequences in three regions of the HIV-1 genes including *gag p17*, *pol protease* (*PR*) and *reverse transcriptase* (*RT*), and *env C2V3*. Almost all HIV-1 subtypes from 1997 to 2007 and 93% of all HIV-1 isolates in 2007 were subtype B. HIV-1 subtypes A, C, D, and F have been detected sporadically since 1997, almost all in Africans and South Americans. The first detected circulating recombinant form (CRF) was CRF01_AE (11-year average annual detection rate, 7.7%). Only two cases of CRF02_AG were detected in 2006. A unique recombinant form (URF) was first detected in 1998 and the total number of URFs reached 25 by year 2007 (average annual detection rate, 4.7%). Eleven of these 25 were detected from 2000 to 2005 and had subtypes AE/B/AE as determined by base sequencing of the *gag p17*, *pol PR* and *RT*, and *env C2V3* genes (average annual detection rate, 3.7%). Unique subtype B has been detected in six cases since 2006. All 17 of these patients were Japanese. Other recombinant HIV-1s have been detected intermittently in eight cases since 1998. During the 11-year surveillance, most HIV-1s in Nagoya, Japan were of subtype B. We expect that subtype B HIV-1 will continue to predominate for the next several years. Active recombination between subtype B and CRF01_AE HIV-1 and its transmission were also shown.

Introduction

THE TOTAL NUMBER OF HIV-1-INFECTED INDIVIDUALS and the prevalence of HIV-1 in Japan were 13,842 and 0.01%, respectively, at the end of 2007, which is far less than the number and prevalence in other Asian countries.[1–6] However, the number of newly infected patients per year was still increasing in Japan in 2007, when it reached 1448. One-hundred and twenty-five (8.6%) new patients were identified in Aichi prefecture in the same year. The total number of HIV-1-infected individuals and the prevalence of HIV in Aichi prefecture in 2007 were 725 and 0.01%, respectively. The number of newly infected individuals is expected to increase further. Therefore, the rapid increase in the number of HIV-1-infected individuals and changes in HIV-1 subtype populations are matters of great concern.

The most prevalent HIV-1 subtype in the world is subtype C (50%) followed by A (12%), B (10%), G (6%), and D (3%).[7,8] In addition, there are circulating recombinant forms (CRFs), such as CRF01_AE, CRF02_AG, and other recombinant viruses. The prevalent HIV-1 subtypes in China, Japan's largest neighbor, are circulating B/C recombinant forms, CRF07_BC, and CRF08_BC, accounting for 50% of the HIV-infected population, and subtype B HIV-1 accounting for 32%.[9] In contrast, the most prevalent HIV-1 strains in Japan are subtype B (83.1%) and CRF01_AE (12.4%) according to the nationwide surveillance.[10] It is clear that the distribution of HIV-1 strains is very different in Japan than in the rest of the world. However, as subtypes were determined from the base sequence of the *pol protease* (*PR*) and *reverse transcriptase* (*RT*) genes, we could not identify recombinant HIV-1 subtypes.

To study the trend of HIV-1 subtypes and the emergence of recombinant HIV-1 subtypes in Nagoya, Japan, we selected the *gag p17*, *env C2V3*, and *PR* to *RT* gene. We determined the base sequences of these genes from 2003; the subtypes of HIV-1 samples from 1997 to 2002 were retrospectively determined by the same method. Finally, this study aimed to clarify the genetic changes in HIV-1 subtypes by monitoring the sequences of HIV-1 subtypes that infected therapy-naive patients between 1997 and 2007.

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TABLE 1. SUBTYPE DISTRIBUTION OF THERAPY-NAIVE HIV-1-INFECTED PATIENTS

	Total	*A*	*B*	*C*	*D*	*F*	*CRF01_AE*	*CRF02_AG*	*Other recombinants*
Number of patients	534	10 (1.9%)	440 (82.4%)	11 (2.1%)	1 (0.2%)	4 (0.7%)	41 (7.7%)	2 (0.4%)	25 (4.7%)
Age									
Average	37	31	37	38	32	24	40	37	35
Range	17–73	20–40	17–73	22–66	—	21–28	20–69	33–41	20–53
$CD4^+$ T cells (cells/μl)									
Average	280	305	278	291	418	468	246	289	264
Range	0–1508	60–1003	0–1508	7–545	—	278–909	2–1200	210–367	1–716
Viral load (copies/ml)									
Average	3.1×10^5	1.8×10^5	3.3×10^5	8.6×10^4	2.5×10^4	2.2×10^4	2.7×10^5	5.1×10^4	2.9×10^5
Range	1.0×10^2–1.3×10^7	4.4×10^2–1.1×10^6	1.0×10^2–1.3×10^7	6.1×10^2–4.3×10^5	—	2.1×10^3–4.5×10^4	7.8×10^2–2.8×10^6	4.4×10^4–5.7×10^4	2.8×10^2–2.0×10^6
Clinical phase[a]									
AC	370	6	302	9	1	4	25	2	21
ARC	37	2	32	0	0	0	3	0	0
AIDS	127	2	106	2	0	0	13	0	4

[a]AC, asymptomatic carrier; ARC, AIDS-related complex.

Materials and Methods

Patients

We enrolled 534 therapy-naive HIV-1-infected patients who initially attended our HIV-1 clinic at Nagoya Medical Center in Nagoya, Japan, between June 1997 and December 2007 and gave their written, informed consent to participate in our study. Our research protocol was approved by the ethical committee in our hospital. The samples of this study were the same ones used for drug resistance testing, which was conducted in almost all therapy-naive individuals at their initial visit. The data, including age, nationality, sexual orientation, number of CD4-positive T cells, and viral load, were obtained from medical records.

Patient coverage by Nagoya Medical Center

Nagoya Medical Center is located in Nagoya City, the capital of Aichi prefecture, and is the central hospital for AIDS treatment and research in the Tokai area (which includes the Aichi, Mie, Gifu, and Shizuoka prefectures).

Amplification of HIV-1 DNA fragments and determinations of DNA sequences

HIV-1 RNA was extracted from plasma samples using a QIAamp viral RNA Mini Kit (QIAGEN, Tokyo, Japan). The DNA fragments were amplified by reverse transcription-nested polymerase chain reaction (RT-nested PCR) using the Superscript one-step RT-PCR system (Invitrogen, Tokyo, Japan) and LA Taq polymerase (Takara, Shiga, Japan). The primers used for DNA amplification were as follows. The *gag* fragment containing the region between *gag p17* and *p24* (codons 1–147 encoding *gag-pol polyprotein*) was amplified by RT-PCR with the primer set of 172A (5′-ATC TCT AGC AGT GGC GCC CGA ACA G-3′) and 173B (5′-CTG ATA ATG CTG AAA ACA TGG GTA T-3′).[11] 174A (5′-CTC TCG ACG CAG GAC TCG GCT TGC T-3′) and 175B(5′-CCC ATG CAT TCA AAG TTC TAG GTG A-3′) were used for nested PCR. The *pol* fragments containing the regions encoding *gag-pol polyprotein* (codons 425–500), *PR* (codons 1–99), and *RT* (codons 1–349) were amplified by RT-PCR with primers of K1 (5′-AAG GGC TGT TGG AAA TGT GG-3′) and U13 (5′-CCC ACT CAG GAA TCC AGG T-3′).[12] K4 (5′-GAA AGG AAG GAC ACC AAA TGA-3′) and U12 (5′-CTC ATT CTT GCA TAT TTT CCT GTT-3′) were used for nested PCR. The *env* fragment containing the region encoding *env C2V3* (codons 249–375) was amplified by RT-PCR with primers of 106A (5′-CAT ACA TTA TTG TGC CCC GGC TGG-3′) and 17B (5′-AGA AAA ATT CCC CTC TAC AAT TAA-3′).[11] 14A (5′-AAT GTC AGC TCA GTA CAA TGC ACA C-3′) and 10B (5′-ATT TCT GGG TCC CCT CCT GAG G-3′) were used for nested PCR. These are in-house primers capable of amplifying the target genes of different HIV-1 subtypes. The *gag*, *pol*, and *env* gene fragments were not amplified in RT-nested PCR using the above-mentioned primers in four, five, and nine cases, respectively. In such cases, different primer sets were used (not shown). Thus, amplified cDNAs were successfully obtained in all cases.

An HIV-1 DNA control, in which reverse transcriptase was omitted in the RT-PCR, was run in parallel with RT-PCR to control for the absence of genomic DNA. The PCR products were purified using the QIAGEN Gel Extraction Kit

(QIAGEN). A labeling reaction for DNA sequencing was performed using the same primers used for the PCR reactions as well as the BigDye terminator v1.1 cycle sequencing kit (Applied Biosystems, Tokyo, Japan), and then labeled DNA fragments were purified on a AutoSeq G-50 column (GE Healthcare, Tokyo, Japan). The samples were analyzed by direct sequencing and electropherograms were obtained using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). DNA sequences were analyzed using Sequencing Analysis V3.7 (Applied Biosystems) and SeqScape V2.5 (Applied Biosystems).

Subtype determination

Sequences were analyzed and grouped into subtypes by phylogenetic tree construction. Phylogenetic analysis using nucleotide sequences of *gag* fragments from 534 therapy-naive patients was performed using the 62 reference HIV-1 sequences derived from the Los Alamos National Laboratory database.[13] The 62 sequences represent all subtypes of the major groups and 18 CRFs. Independent analysis was done using both *pol* fragments and *env* fragments. Sequences were aligned using CLUSTAL W software in MEGA software version 3.1. Evolutionary distances were calculated using the same software. Phylogenetic trees were constructed by the neighbor-joining method based on distances calculated with Kimura's two-parameter algorithm. The reliabilities of the branching patterns were tested by bootstrap analysis with 1000 replicates.

Results

HIV-1 subtypes in the last 11 years in Nagoya

Subtype B was the most prevalent subtype, identified in 440 out of 534 patients (82.4%, Table 1 and Fig. 1). The largest number, 388, was found in Japanese and the next largest, 45, in South Americans (Table 2). Of the total number, 319 were identified in Japanese men who have sex with men (MSM) and 14 in South American MSM. Of note, the 72.5% infected with subtype B HIV-1 were Japanese MSM. Infection with subtypes A, C, D, and F was detected in 10 individuals (seven Africans, one East European, and two Japanese), 11 individuals (two Africans, two South Americans, two Southwest

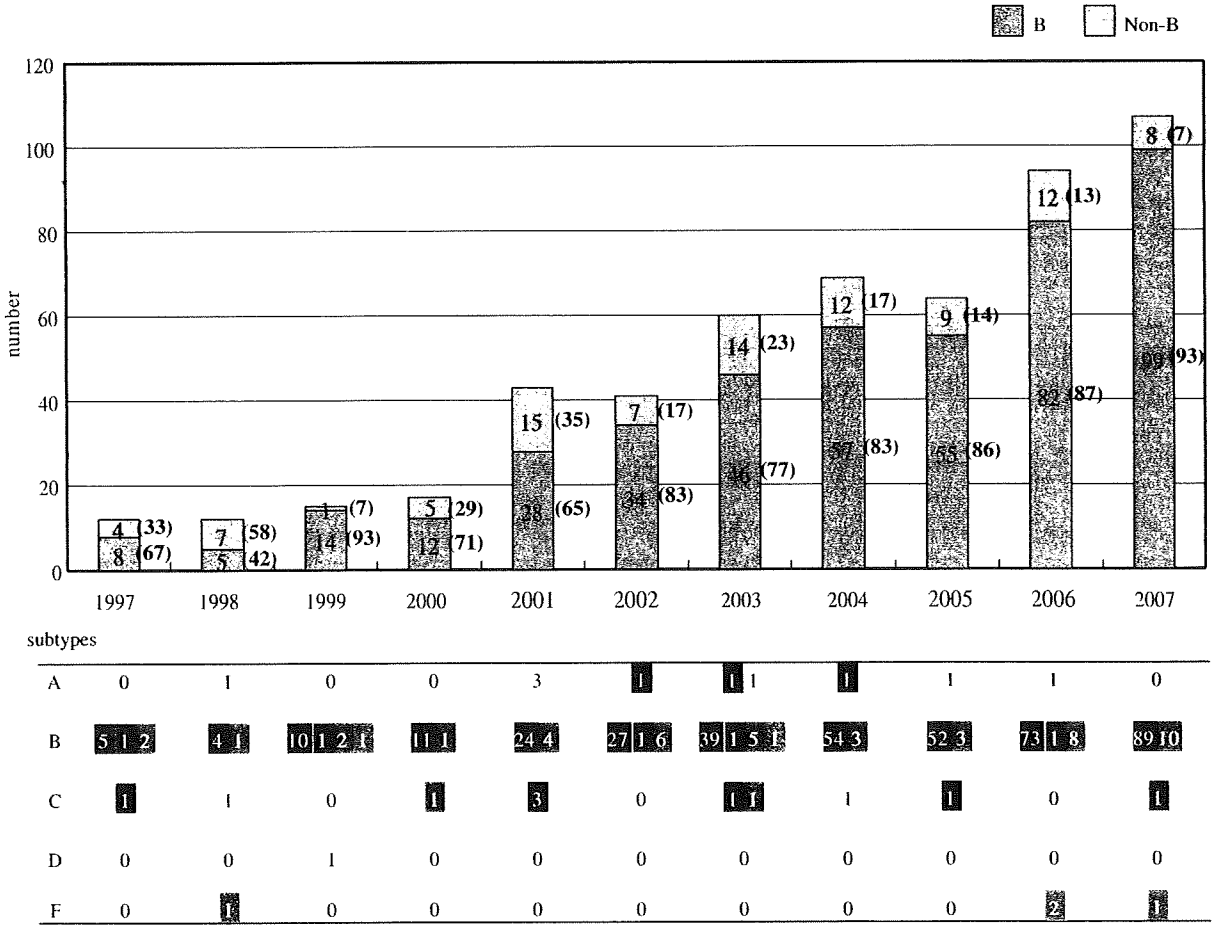


FIG. 1. Subtypes of HIV-1 found in therapy-naive infected patients in Nagoya Medical Center from 1997 to 2007 (upper panel). The numbers of patients (bars) and their proportions (%, in parentheses) are shown. The numbers of patients and their nationalities (indicated by a color: Japan, red; East Asia, green; Southwest Asia, blue; South America, orange; North America, pink; Eastern Europe, black; Africa, uncolored) are given in the lower panel.

TABLE 2. NATIONALITIES AND SEXUAL ORIENTATIONS OF THERAPY-NAIVE HIV-1-INFECTED PATIENTS BY SUBTYPES[a]

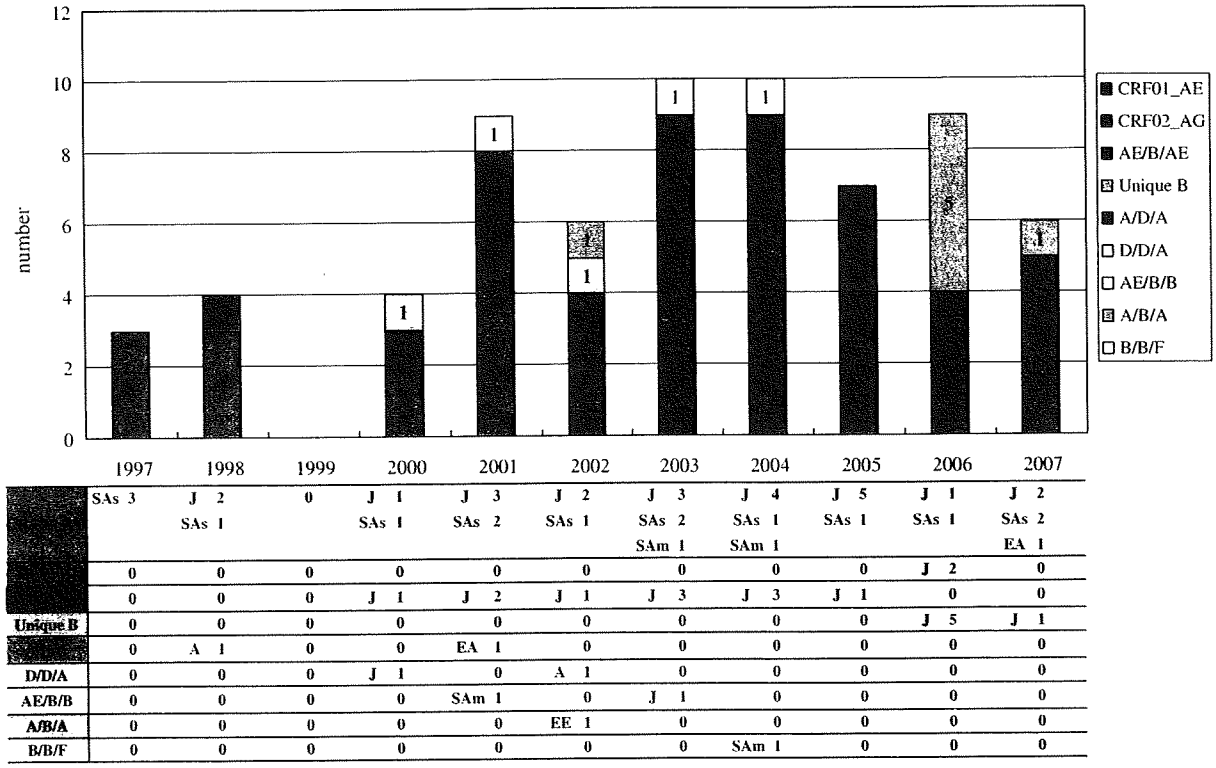
	Total	*A*	*B*	*C*	*D*	*F*	*CRF01_AE*	*CRF02_AG*	*Other recombinants*
Nationalities									
Japan	439 (420, 340)	2 (0, 0)	388 (381, 319)	5 (1, 1)	0	0	23 (20, 2)	2 (0, 0)	19 (18, 18)
East Asia	6 (4, 4)	0	4 (4, 4)	0	0	0	1 (0, 0)	0	1 (0, 0)
Southwest Asia	18 (9, 0)	0	1 (0, 0)	2 (1, 0)	0	0	15 (8, 0)	0	0
East Europe	2 (0, 0)	1 (0, 0)	0	0	0	0	0	0	1 (0, 0)
North America	2 (2, 2)	0	2 (2, 2)	0	0	0	0	0	0
South America	55 (36, 15)	0	45 (31, 14)	2 (1, 0)	0	4 (1, 0)	2 (1, 0)	0	2 (2, 1)
Africa	12 (9, 0)	7 (5,0)	0	2 (1, 0)	1 (1, 0)	0	0	0	2 (2, 0)

[a]The left and right numbers in parentheses are the numbers of males and MSMs, respectively.

Asians, and five Japanese), one African, and four South Americans, respectively. In foreigners, this distribution of HIV-1 subtypes represents the distribution of subtypes commonly found in the immigrants' region of origin.[14–20] However, subtypes A and C were detected mostly in Japanese females who seemed to have acquired the infection from foreign partners in Japan.

As for circulating recombinant forms (CRFs), CRF01_AE was detected in 41 individuals (7.7%), including 23 Japanese (18 non-MSM, two MSM, and three females) and 18 foreigners (nine non-MSM and nine females). Considering that only four individuals (two Japanese males, one Japanese female, and one South Asian female) were intravenous drug users, CRF01_AE has mainly spread through heterosexual contact in Nagoya. CRF02_AG was detected in two individuals (0.4%) in 2006 (Fig. 2).

Among other recombinant forms, the most frequent was AE/B/AE, found in 11 Japanese MSM. A unique subtype B HIV-1 strain that possessed subtype D fragments in the *gag* and *env* regions was also detected in six Japanese MSM. As for other recombinants, A/D/A, D/D/A, and AE/B/B were each identified in two individuals and A/B/A and B/B/F were each identified in one. Thirteen intravenous drug users (2.4%) were included: two male Japanese with subtype B, one female



	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007
	SAs 3	J 2 SAs 1	0	J 1 SAs 1	J 3 SAs 2	J 2 SAs 1	J 3 SAs 2 SAm 1	J 4 SAs 1 SAm 1	J 5 SAs 1	J 1 SAs 1	J 2 SAs 2 EA 1
	0	0	0	0	0	0	0	0	0	J 2	0
	0	0	0	J 1	J 2	J 1	J 3	J 3	J 1	0	0
Unique B	0	0	0	0	0	0	0	0	0	J 5	J 1
	0	A 1	0	0	EA 1	0	0	0	0	0	0
D/D/A	0	0	0	J 1	0	A 1	0	0	0	0	0
AE/B/B	0	0	0	0	SAm 1	0	J 1	0	0	0	0
A/B/A	0	0	0	0	0	EE 1	0	0	0	0	0
B/B/F	0	0	0	0	0	0	0	SAm 1	0	0	0

FIG. 2. Recombinant HIV-1s detected in therapy-naive patients in Nagoya Medical Center (upper panel). The bars indicate the number of recombinant HIV-1s detected from 1997 to 2007. The number of patients and their nationalities (indicated by J, Japan; EA, East Asia; SAs, Southwest Asia; SAm, South America; A, Africa; NA, North America; EE, Eastern Europe) are shown in the lower panel.

Japanese with CRF01_AE, four MSM with a unique subtype B, two male South Americans with subtype B, and two male and one female Southwest Asians with CRF01_AE.

The change of HIV-1 detection rates

The rate of subtype B detection, the most frequently detected HIV-1 subtype in the Nagoya Medical Center, increased to 93% in 2007 (Fig. 1). During this period, the absolute number of individuals with subtype B HIV-1 also increased. On the other hand, the number of individuals infected with non-B HIV-1 strains fluctuated between 7 and 15 after the year 2000. Major subtypes A, C, D, and F were sporadically detected (only two or three cases per year since 2004). The most frequently detected circulating recombinant HIV-1 was CRF01_AE, which was detected every year from 1997 to the present, except for 1999 (Fig. 2). About half of these infections were in Japanese and the other half were in Southwest Asians. No increase in the number of infections by this virus has been observed. The increase in the number of HIV-1-infected cases, especially of subtype B, during the past 11 years reflected the scaling up of clinical services as well as the increase in HIV-1 transmission.

Active recombination between subtype B and CRF01_AE HIV-1

The A/D/A recombinant was first detected in 1998 (Fig. 2), and unique recombinant forms (URFs) have been detected in all years except 1999. The AE/B/AE recombinant, the most frequently detected URF, had been detected between 2000 and 2005, and the accumulated number of cases with this virus was 11. A unique subtype B HIV-1 was detected in five patients and one patient in 2006 and 2007, respectively.

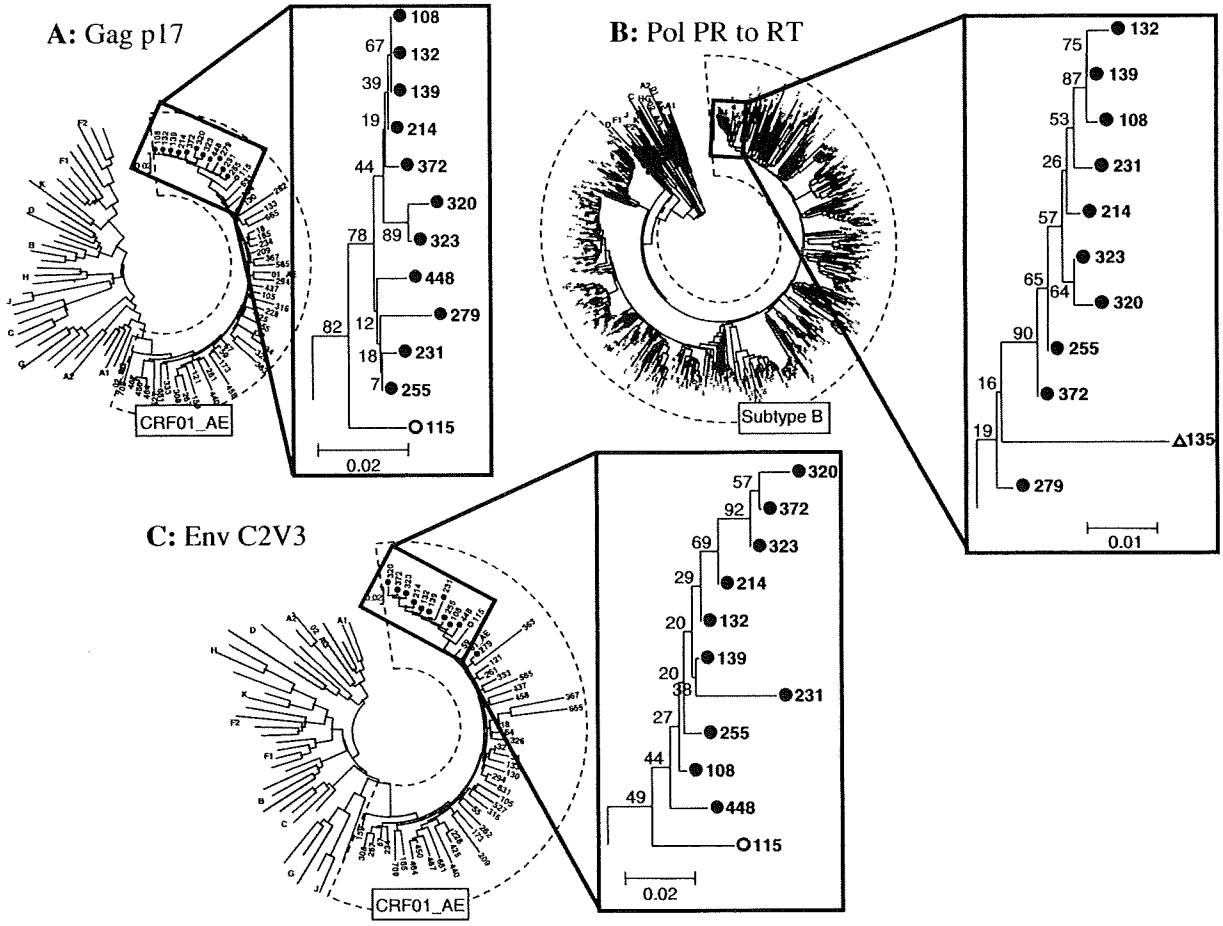


FIG. 3. Phylogenetic tree analyses of 11 recombinant AE/B/AE HIV-1s. Trees were constructed using nucleotide base sequences of *gag p17* (**A**), *pol PR* to *RT* (**B**), and *env C2V3* (**C**) gene regions. The base sequences of the *gag p17* and *env C2V3* genes of 41 CRF01_AE HIV-1s and those of the *pol PR* to *RT* gene of 440 subtype B HIV-1s in this study (Table 1) as well as 39 reference sequences obtained from the Los Alamos database were used for these analyses. Clustered AE/B/AE recombinant HIV-1s indicated by red, blue, and green circles are framed. Red circles represent HIV-1s that form a cluster in all trees. Green circles represent HIV-1s that form a cluster only in trees with *gag* and *env* genes. Blue circles represent HIV-1s that form a cluster only in trees with *gag* and *pol* genes. Candidate HIV-1s with subtype B or CRF01_AE accounting for AE/B/AE recombinant formation are depicted by open triangles (**B**) and open circles (**A** and **C**), respectively.

The detection of one A/D/A, one D/D/A, two AE/B/B, one A/B/A, and one B/B/F was sporadic from 2001 to 2004. The A/D/A, D/D/A, AE/B/B, A/B/A, B/B/F, and unique B recombinants may have been brought from foreign countries because no candidate sequences indicating the origin of A, D, F, and CRF01_AE have been found. Of course, patients infected with these recombinant viruses may have been partners in a sexual network with less access to medical care. Nine viruses of the AE/B/AE recombinant form (represented with red circles in Fig. 3) were clustered in three separate phylogenetic analyses of the *gag p17*, *env C2V3*, and *pol PR* to *RT* genes, indicating that they were closely related. In addition, CRF01_AE of patient 115 was positioned in the same cluster of *gag* and *env* sequences mentioned above, and subtype B of patient 135 in the same cluster of *pol* sequence, suggesting this AE/B/AE recombinant form may be originated from these two viruses.

Discussion

In this study, the emergence and transmission of recombinant HIV-1s were analyzed in detail by determining base sequences of major segments of three genes: *gag p17*, *pol PR* to *RT*, and *env C2V3*. The subtypes of HIV-1 strains prevalent in the Nagoya Medical Center for the past 11 years were B (82.4%), CRF01_AE (7.7%), C (2.1%), A (1.9%), F (0.7%), CRF02_AG (0.4%), D (0.2%), and other recombinant forms (4.7%). This result was similar to the result of a nationwide study (using only the *pol PR* to *RT* gene region to determine subtypes[10]), which found that only 5% of the total cases were due to recombinant forms. Worldwide, subtype C is the most prevalent HIV-1 subtype (50% of all cases).[8,9] This means the situation in Nagoya, Japan is quite different from that in other parts of the world, especially from that in Africa.

The recombinant HIV-1s, in addition to CRF01_AE and CRF02_AG, include 11 AE/B/AE, six unique subtype B recombinants possessing short D fragments in *gag* and *env* genes, two A/D/A, two D/D/A, two AE/B/B, one A/B/A, and one B/B/F. Among these seven, all except AE/B/AE appear to be immigrant types. Only the AE/B/AE type seems to be a newly emerged recombinant in our area. As subtype B was the predominant HIV-1 subtype and CRF01_AE was the second-most prevalent HIV-1 recombinant in Nagoya, we have speculated that a novel recombinant HIV-1 between subtype B and CRF01_AE will emerge in this area. The methodology for subtype determination using the base sequences of *gag p17*, *pol PR* to *RT*, and *env C2V3* genes is useful at the moment; however, to clarify the gene structure of these recombinant viruses, analysis of the full sequence and then computational analyses are required. Use of this approach revealed that the gene structure of AE/B/AE type recombinants found in this study is novel and differs from that of CRF15_01B, CRF33_01B, and CRF34_01B (i.e., CRFs resulting from recombination between subtype B and CRF01_AE HIV-1 found in Malaysia and Thailand).[21–23] The finding of two viruses with genes for both subtype B and CRF01_AE, which are the candidate origins of this AE/B/AE recombinant, supported our hypothesis of emerging recombinants. The number of HIV-1-infected individuals as well as the frequency of drug-resistant HIV-1 in therapy-naive individuals have increased, strongly implying that this increasing tendency will continue in Nagoya, Japan.[24,25] We will pursue this kind of surveillance to obtain information needed for suppressing the spread of HIV-1 infection.

Sequence Data

The base sequences of HIV-1 subtypes are registered in the DNA databank of Japan (DDBJ) as AB442228–AB443428 and AB356098–AB356499.

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Disclosure Statement

No competing financial interests exist.

References

1. Japanese Foundation of AIDS Prevention: http://www.jfap.or.jp/english/index.htm.
2. Wu Z, Sun X, Sullivan SG, and Detels R: HIV testing in China. Science 2006;312:1475–1476.
3. Wu Z, Sullivan SG, Wang Y, *et al.*: Evolution of China's response to HIV/AIDS. Lancet 2007;369:679–690.
4. Steinbrook R: HIV in India—a complex epidemic. N Engl J Med 2007;356:1089–1093.
5. Wirachsilp P, Kantakamalakul W, Foongladda S, *et al.*: Surveillance of subtype and genetic variation of the circulating strains of HIV-1 in Thailand. Southeast Asian J Trop Med Public Health 2007;38:814–827.
6. Chang SY, Sheng WH, Lee CN, *et al.*: Molecular epidemiology of HIV type 1 subtypes in Taiwan: Outbreak of HIV type 1 CRF07_BC infection in intravenous drug users. AIDS Res Hum Retroviruses 2006;22:1055–1066.
7. Hemelaar J, Gouws E, Ghys PD, and Osmanov S: Global and regional distribution of HIV-1 genetic subtypes and recombinants in 2004. AIDS 2006;20:W13–23.
8. McCutchan FE: Global epidemiology of HIV. J Med Virol 2006;78:S7–S12.
9. Zhong P, Pan Q, Ning Z, *et al.*: Genetic diversity and drug resistance of human immunodeficiency virus type 1 (HIV-1) strains circulating in Shanghai. AIDS Res Hum Retroviruses 2007;23:847–856.
10. Gatanaga H, Ibe S, Matsuda M, *et al.*: Drug-resistant HIV-1 prevalence in patients newly diagnosed with HIV/AIDS in Japan. Antiviral Res 2007;75:75–82.
11. Kato K, Shiino T, Kusagawa S, *et al.*: Genetic similarity of HIV type 1 subtype E in a recent outbreak among injecting drug users in northern Vietnam to strains in Guangxi Province of southern China. AIDS Res Hum Retroviruses 1999;15:1157–1168.
12. Ibe S, Shibata N, Utsumi M, and Kaneda T: Selection of human immunodeficiency virus type 1 variants with an insertion mutation in the $p6^{gag}$ and $p6^{pol}$ genes under highly active antiretroviral therapy. Microbiol Immunol 2003; 47:71–79.
13. Los Alamos HIV Sequence Database: http://www.hiv.lanl.gov/content/sequence/HIV/mainpage.html.
14. Katzenstein D: Diversity, drug resistance, and the epidemic of subtype C HIV-1 in Africa. J Infect Dis 2006;194:S45–50.

15. Herbeck JT, Lyagoba F, Moore SW, *et al.*: Prevalence and genetic diversity of HIV type 1 subtypes A and D in women attending antenatal clinics in Uganda. AIDS Res Hum Retroviruses 2007;23:755–760.
16. Baker CA, Bousheri S, Ssewanyana I, *et al.*: HIV subtypes distribution and implication for antiretroviral treatment in a Ugandan population. J Int Assoc Phys AIDS Care 2007; 6:260–263.
17. Trask SA, Derdeyn CA, Fideli U, *et al.*: Molecular epidemiology of human immunodeficiency virus type 1 transmission in a heterosexual cohort of discordant couples in Zambia. J Virol 2002;76;397–405.
18. Bello G, Guimarães ML, and Morgado MG: Evolutionary history of HIV-1 subtype B and F infections in Brazil. AIDS 2006;20:763–768.
19. Eyer-Silva WA, Couto-Fernandez JC, and Morgado MG: Molecular epidemiology of HIV type 1 in inner Rio De Janeiro State, Brazil. AIDS Res Hum Retroviruses 2007;23:303–308.
20. Varella RB, Ferreira SB, de Castro MB, Zalis MG, and Tavares MD: Human immunodeficiency virus type 1 protease and reverse transcriptase mutation patterns among treatment-naive patients in different stages of infection in Rio de Janeiro, Brazil. J Med Virol 2007;79:1033–1039.
21. Tovanabutra S, Watanaveeradej V, Viputtikul K, *et al.*: A new circulating recombinant form, CRF15_01B, reinforces the linkage between IDU and heterosexual epidemics in Thailand. AIDS Res Hum Retroviruses 2003;19:561–567.
22. Tee KK, Li XJ, Nohtomi K, Ng KP, Kamarulzaman A, and Takebe Y: Identification of a novel circulating recombinant form (CRF33_01B) disseminating widely among various risk populations in Kuala Lumpur, Malaysia. J Acquir Immune Defic Syndr 2006;43:523–529.
23. Tovanabutra S, Kijak GH, Beyrer C, *et al.*: Identification of CRF34_01B, a second circulating recombinant form unrelated to and more complex than CRF15_01B, among injecting drug users in northern Thailand. AIDS Res Hum Retroviruses 2007;23:829–833.
24. Ibe S, Hotta N, Takeo U, *et al.*: Prevalence of drug-resistant human immunodeficiency virus type 1 in therapy-naive patients and usefulness of genotype testing. Microbiol Immunol 2003;47:499–505.
25. Ibe S, Hattori J, Fujisaki S, *et al.*: Trend of drug-resistant HIV type 1 emergence among therapy-naïve patients in Nagoya, Japan: An 8-year surveillance from 1999 to 2006. AIDS Res Hum Retroviruses 2008;24:7–14.

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Human Herpesvirus 8 DNA Load in the Leukocytes Correlates with the Platelet Counts in HIV Type 1-Infected Individuals

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Abstract

Human herpes virus 8 (HHV-8) is known to be reactivated in immunocompromised situations and it is associated with Kaposi's sarcoma (KS) and some hematological diseases. The aim of this study was to analyze the effect of HHV-8 on HIV-1 infection, especially on thrombocytopenia complicated with HIV infection. The HHV-8 DNA load was determined by a quantitative real-time PCR, using leukocytes from 125 HIV-1-infected individuals. HHV-8 DNA was detected in 37 individuals. The increased HIV-1 load and reduced percentage of CD4-positive T cells were significantly associated with the presence of HHV-8. The prevalence and load for HHV-8 are higher in patients with KS than in patients without KS, but the difference is not significant. The increased HHV-8 DNA load was significantly correlated with thrombocytopenia, and platelet counts were significantly lower in individuals with HHV-8 than in individuals without HHV-8. We also obtained the negative correlations between changes in platelet counts and changes in HHV-8 DNA loads. The association between thrombocytopenia and HHV-8 has never been reported previously, apart from some case reports of Castleman's disease and KS. Various cytokines or chemokines are produced by HHV-8-infected cells, some of which have been reported to inhibit hematopoiesis. This may be one of the mechanisms by which HHV-8 infection induces thrombocytopenia. These results indicate that HHV-8 DNA in leukocytes may provide useful information for the assessment of the clinical appearance of HIV-1 infection.

Introduction

HHV-8, A NEW MEMBER OF THE GAMMAHERPESVIRINAE, was identified as the etiologic agent of Kaposi's sarcoma (KS). The main transmission routes of human herpes virus 8 (HHV-8) seem to be sexual contact,[1] but transmission by saliva,[2] blood products, and organ graft[3,4] has also been proposed. The distribution of HHV-8 is related to a combination of geographic and behavioral risk factors. Serological studies have shown that HHV-8 seroprevalence is high in Africa and the Middle East and low in Europe and the United States. In Japan, it is reported that HHV-8 seroprevalence among healthy controls is 0.2–1.4% and HHV-8 seroprevalence among HIV-1-positive homosexual men is 11.6–63.6%.[5,6]

HHV-8 can infect circulating B cells, monocytes, macrophages, T cells, and KS-like spindle cell progenitors,[7–9] and usually persists in a latent state in these cells. The reactivation of this latent HHV-8 infection can be induced by a number of conditions, including superinfection by other viruses, stress, chronic illnesses, malignancies, and immunosuppressive disorders, such as HIV infection. HHV-8 contains more than 80 open reading frames, including several homologues of oncogenes, cytokine, and cytokine response genes. During latent and lytic infection, some viral genes are expressed and play a causative role in the genesis of some diseases, such as AIDS and non-AIDS-related KS, multicentric Castleman's disease, body cavity-based lymphoma, and some lymphoproliferative diseases. Our previous study reported a case of Castleman's disease with HIV-1 infection in which repeated episodes of thrombocytopenia were correlated with an increase in HHV-8 DNA loads in leukocytes.[10] The aim of this study was to determine the prevalence and loads of HHV-8 DNA in peripheral blood leukocytes in HIV-1-positive individuals and to investigate the correlation with the clinical appearance of HIV-1 infection, especially with thrombocytopenia.

Materials and Methods

Patients and samples

All consecutive HIV-1-infected patients who attended Kyushu Medical center between April 2005 and August 2006

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were eligible for this study. The exclusion criteria were HCV infection, active hepatitis, and treatment for antiherpes therapy at the time of sampling. Nine HIV-1-positive individuals were analyzed longitudinally before and after the initiation of antiretroviral therapy (ART). For controls, 12 HIV-1-seronegative patients with autoimmune thrombocytopenia and 17 HIV-1-seronegative healthy volunteers were sampled for baseline comparisons. Informed consent for blood sampling was obtained from all participants. The study was conducted according to the ethical guidelines of the hospital and was approved by an authorized representative of the hospital. EDTA-treated blood was taken from the subjects, and leukocytes were collected after removing red blood cells with hemolysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA). Cell pellets were stored at −20°C until use.

Real-time quantitative PCR

DNA was extracted from the cell pellets using a QIAamp Blood Mini kit (QIAGEN Inc., Tokyo, Japan). Real-time PCR was conducted with the LineGene33 (BioFlux, Tokyo, Japan) using Premix Ex Taq (TAKARA, Shiga, Japan). As an internal control measurement, to normalize for input DNA, copy numbers of ß$_2$-microglobulin (ß$_2$M) were determined in every sample tested. The primers used for amplification were as follows: HHV-8 forward, 5′-CCTCTGGTCCCCATTCATTG-3′, and reverse, 5′-CGTTTCCGTCGTGGATGAG-3′, and probe 5′-FAM-CCGGCGTCAGACATTCTCACAACC-TAMRA-3′;[11] Epstein–Barr virus (EBV) forward, 5′-CGGAAGCCCTCTGGACTTC-3′, and reverse, 5′-CCCTGTTTATCCGATGGAATG − 3′, and probe 5′-FAM-TGTACACGCACGAGAAATGCGCC-TAMRA 3′;[12] ß ₂M forward, 5′-CAGCAAGGACTGGTCTTTCTATCTCT-3′, and reverse, 5′-ACCCCACTTAACTATCTTGG-3′, and probe 5′-FAM-CACTGAAAAAGATGAGTATGCCTGCCGTGT-TAMRA-3′.[13] Standards were obtained by amplification of a control sample in a polymerase chain reaction (PCR) reaction using the same primers. The data were normalized as copies/10^6 cells by measuring copy numbers of the ß$_2$M gene, since two ß$_2$M copy numbers correspond to one cell. The lower limit of detection was defined as 1 copy/10^6 cells.

Statistical analyses

Frequency analysis was performed using Fisher's exact test for 2×2 tables. The viral DNA copy numbers of HHV-8, EBV, and HIV-1 were $\log_{10}$ transformed and compared between different groups by means of the Mann–Whitney *U* test. The associations between them were determined using an analysis of covariance (ANCOVA), with EBV-DNA and HIV-RNA as covariates, where HHV-8 DNA was adjusted on the bases of EBV-DNA and HIV-RNA. Spearman's rank correlation coefficient was used to compare changes in platelet counts and changes in HHV-8 DNA loads. A partial correlation was used to assess this relationship, while controlling for any changes in HIV-RNA and EBV-DNA.

Results

Subjects characteristics

A total of 125 patients fulfilled the inclusion criteria. Demographic and clinical characteristics of the subjects included in this study are shown in Table 1. There were 8 women and 117 men, 112 of whom were men who have sex with men; the others were heterosexual. These patients were ranged from 20 to 69 years of age (mean age, 37.8 years). Of these, 58 received ART and 67 remained untreated. The duration of ART was 3 months to 9.5 years (mean 3.25 years). Nine HIV-1-positive individuals were analyzed longitudinally before and after the initiation of ART. Among 125 HIV-1-positive subjects included in our study, we evaluated 97 subjects for the prevalence of splenomegaly by means of an abdominal ultrasound examination; 23 subjects were thus found to have splenomegaly.

HHV-8 qualitative and quantitative DNA analysis

The presence and load of HHV-8 DNA were investigated in 125 HIV-1-positive subjects and 17 HIV-1-negative healthy controls (Fig. 1). HHV-8 DNA was detected in 37 of 125 (29.6%) leukocyte samples from HIV-1-positive subjects, with values ranging from 2 to 91,171 copies/10^6 leukocytes. HHV-8 DNA was not detected in any healthy controls.

Relationship between HHV-8 DNA and KS

HHV-8 DNA was detected in three of five (60%) HIV-infected subjects with KS, whereas they were found in 34 of 120 (28.3%) subjects without KS ($p = 0.15$). Figure 2 shows that the HHV-8 DNA load of subjects with KS to be higher than that of the subjects without KS, but not significantly so.

The correlation between HHV-8 DNA and HIV-1-related immunovirological parameters

The HIV-RNA load in the serum of HHV-8 DNA-positive subjects was significantly higher than that of HHV-8 DNA-negative subjects (Fig. 3a). In addition, the correlation between HHV-8 DNA and hypergammaglobulinemia (the percentage of gammaglobulin is more than normal range) was examined, which is often observed in HIV-1-positive subjects. The HHV-8 DNA load in leukocytes in subjects with

TABLE 1. DEMOGRAPHIC AND CLINICAL CHARACTERISTICS OF 125 HIV-POSITIVE SUBJECTS[a]

	Total	*Pretreatment of ART*	*Treatment of ART*	p
N (male/female)	125 (117/8)	67 (63/4)	58 (54/4)	
Age (years)	37.8 ± 10.4	34.3 ± 8.2	42.1 ± 11.2	<0.001
CD4 (cells/ml)	396 ± 218	387 ± 226	407 ± 211	0.616
Viral load (copies/ml) ($\log_{10}$)		4.36 + 0.86	Under detection limit	
Duration of ART (years)		0	3.25 ± 2.68	

[a]The mean values ± SE are shown. Pretreatment of ART versus treatment of ART.

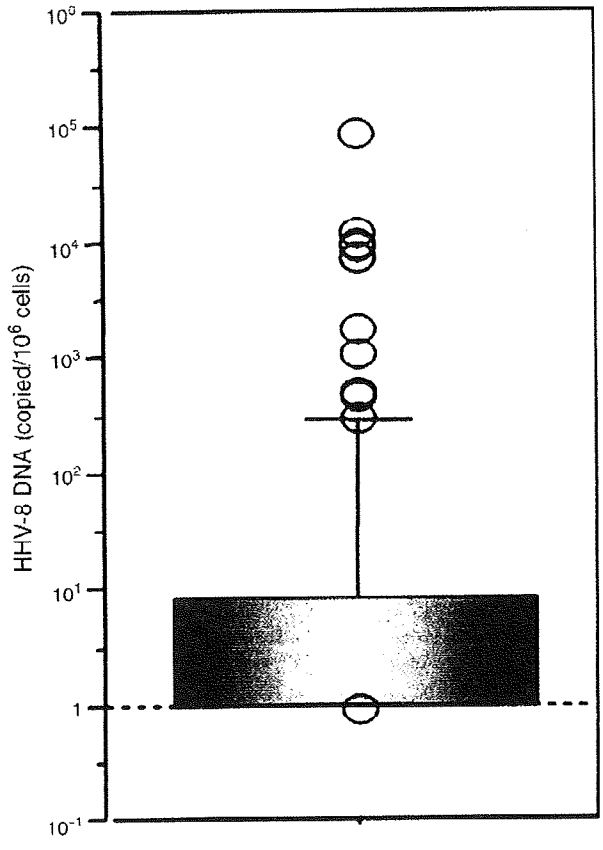

FIG. 1. Quantification of HHV-8 DNA by real-time PCR. Log_{10}-transformed HHV-8 copy numbers per 10^6 leukocytes are shown. The bar shows the mean and standard deviations. The dotted line shows the detection limit of the assay.

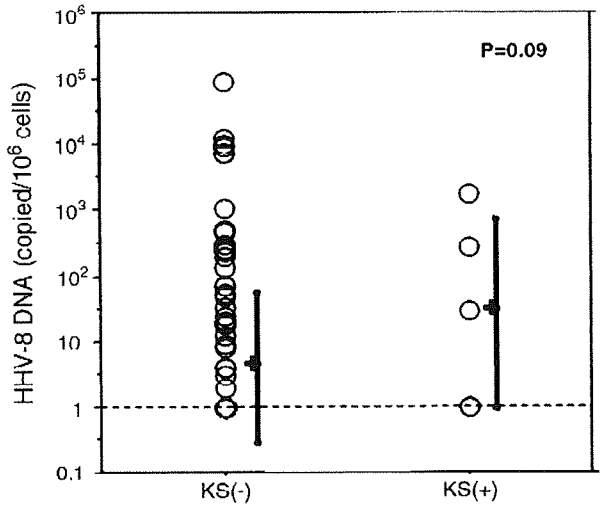

FIG. 2. HHV-8 DNA loads among the HIV-1-positive subjects with or without KS. Log_{10}-transformed HHV-8 copy numbers per 10^6 leukocytes are shown. The bar shows the mean and standard deviations. *p*-values were estimated by the Mann–Whitney *U* test. The dotted line shows the detection limit of the assay.

hypergammaglobulinemia was significantly higher than in subjects without hypergammaglobulinemia (Fig. 3b). HHV-8 DNA was detected in 24 of 44 (54.5%) HIV-infected subjects with hypergammaglobulinemia, whereas it was found in 13 of 81 (16.0%) subjects without hypergammaglobulinemia ($p < 0.0001$). We could not find any significant correlation between HHV-8 DNA loads and CD4-positive T cell counts.

The effect of ART on HHV-8 DNA

HHV-8 DNA load in leukocytes in subjects with ART was significantly lower than in subjects without ART (Fig. 4a). HHV-8 DNA was detected in 12 of 58 (20.7%) HIV-infected subjects with ART, whereas it was found in 25 of 67 (37.3%) subjects without ART (p 0.042). There was no difference in the duration of ART between HHV-8 DNA-negative and HHV-8 DNA-positive subjects with ART (3.161 ± 2.764 vs. 3.375 ± 2.439 years, $p = 0.624$). HHV-8 DNA loads (log_{10}) per 10^6 leukocytes in subjects with a short duration of ART (<6 months) were higher than in subjects with a long duration of ART (≥6 months), but the difference was not statistically significant (0.758 ± 0.447 vs. 0.323 ± 0.111, $p - 0.309$).

Longitudinal analysis of HHV-8 DNA loads after initiation of ART

The longitudinal profiles of HHV-8 DNA loads were examined in nine subjects after initiation of ART (Fig. 4b). Within 1 month after starting ART, HHV-8 DNA transiently increased in three patients (Fig. 4b, black straight lines); in two of the three patients, KS had progressed transiently. These findings show the reactivation of HHV-8 by initiation of ART. Three months after the initiation of ART, all nine patients had achieved and maintained the decreased levels of HHV-8 DNA.

The influence of EBV on HHV-8 infection

Like HHV-8, EBV is lymphotropic and is a member of the gammaherpesvirinae; it is reactivated by HIV-1 infection. Therefore, the influence of EBV on HHV-8 infection was investigated. EBV-DNA loads (log_{10}) per 10^6 leukocytes were significantly higher in HHV-8 DNA-positive subjects (0.69 + 0.11 vs. 1.46 + 0.21, $p = 0.0007$) and EBV DNA was detected in 32 of 88 (36.4%) HHV-8-negative subjects, whereas they were found in 25 of 37 (67.5%) HHV-8-positive subjects ($p - 0.002$).

Relationship between HHV-8 DNA and thrombocytopenia

Figure 5 shows a case of Castleman's disease in which the platelet counts changed in inverse association with HHV-8 DNA in the leukocytes. Therefore, the association between platelet counts and HHV-8 DNA in leukocytes was examined. First, the changes in platelet counts and HHV-8 DNA were analyzed in the nine subjects before and after the initiation of ART (Fig. 6). Both the decrease in HHV-8 DNA and the

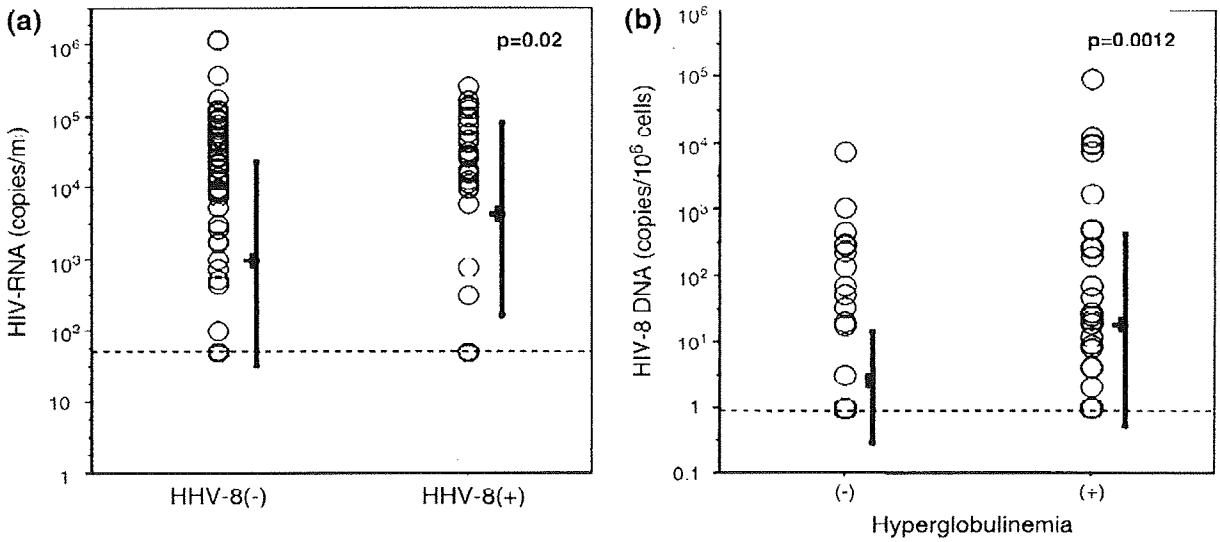

FIG. 3. The correlation between HHV-8 DNA and HIV-1-related immunovirological parameters. (a) HIV-RNA loads among the HIV-1-positive subjects with or without HHV-8 DNA. Log_{10}-transformed HIV-1 copy numbers/ml of serum are shown. The bar shows the mean and standard deviation. p-values were estimated by the Mann–Whitney U test. (b) HHV-8 DNA loads among the HIV-1-positive subjects with or without hypergammaglobulinemia. HHV-8 copy numbers per 10^6 leukocytes are shown. The bar shows the mean and standard deviations. p-values were estimated by the Mann–Whitney U test. The dotted line shows the detection limit of the assay.

increase in platelet counts were significant. Furthermore, the correlation between changes in platelet counts and changes in HHV-8 DNA was obtained (Spearman; $r = -0.783$, $p = 0.0267$). Patients with HIV infection sometimes develop thrombocytopenia, and EBV is also reported to cause mild thrombocytopenia. Therefore, partial correlations were also used to assess this relationship while controlling for EBV-DNA and HIV-RNA, and the correlation between the change in HHV-8 DNA and platelet counts was obtained (partial correlation coefficient; $r = -0.733$, $p = 0.0219$).

Next, all 125 subjects with HIV infection were analyzed. There was no significant difference between the group with

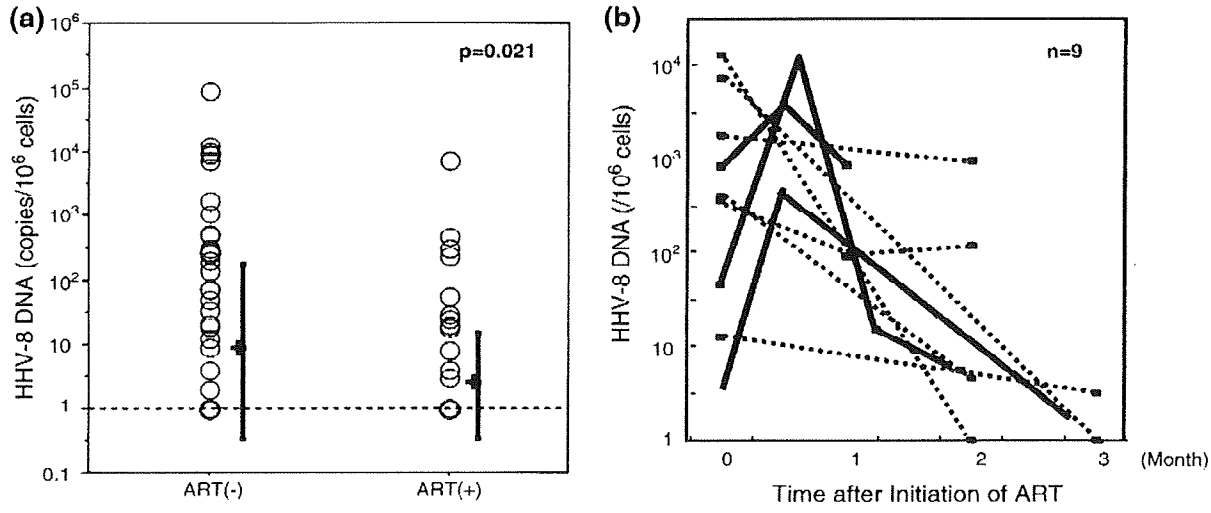

FIG. 4. The effect of ART on HHV-8 DNA. (a) HHV-8 DNA load in PBMCs in subjects with or without ART. HHV-8 copy numbers per 10^6 leukocytes are shown. The bar shows the mean and standard deviation. p-values were estimated by the Mann–Whitney U test. (b) HHV-8 DNA load after initiation of ART. HHV-8 DNA loads of the nine subjects were measured at the indicated time points. The dotted line shows the detection limit of the assay.

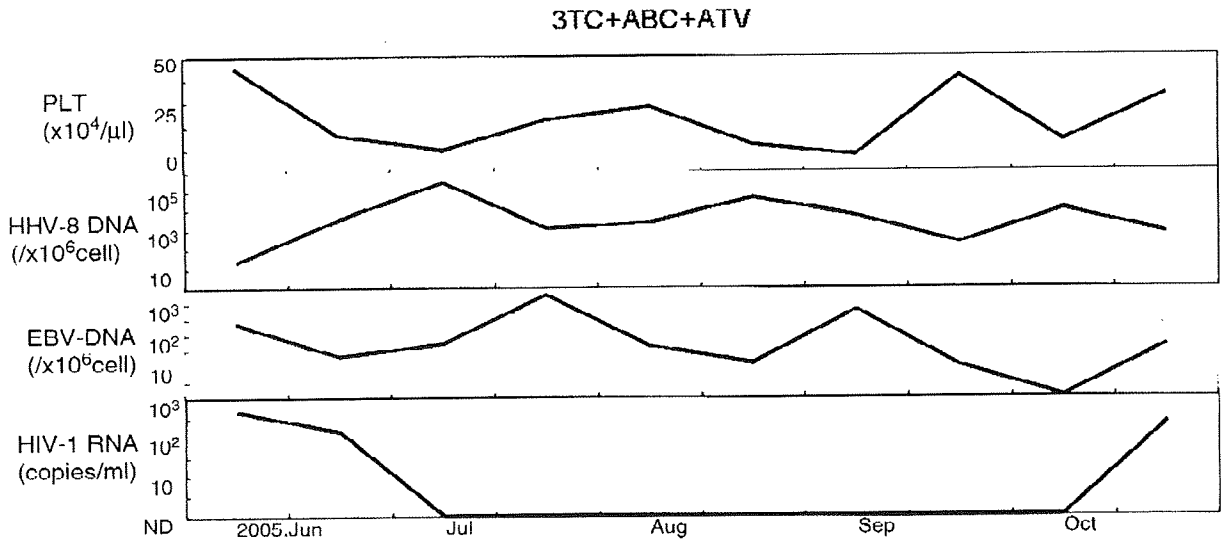

FIG. 5. Time course of the case of Castleman's disease with HIV-1 infection. Platelet counts, $\log_{10}$-transformed HHV-8 and EBV copy numbers per 10^6 leukocytes, and $\log_{10}$-transformed HIV-1 copy numbers/ml of serum are shown. The data were excerpted from Minami and Yamamoto.[10] PLT, platelet; HHV-8, human herpes virus 8, EBV, Epstein–Barr virus, 3TC, lamivudine; ABC, abacavir; ATV, atazanavir.

thrombocytopenia and the group without thrombocytopenia in terms of the number of subjects with ART, the duration of ART, and the prevalence of subjects with splenomegaly (with thrombocytopenia vs. without thrombocytopenia; 30.8% vs. 22.6%, $p = 0.520$). Similarly, no significant difference was observed between the group with HHV-8-DNA and the group without HHV-8-DNA in terms of the duration of ART and the prevalence of subjects with splenomegaly (with HHV-8 vs. without HHV-8; 26.7% vs. 22.4%, $p = 0.647$). The effects of HHV-8 DNA in leukocytes on platelet counts were examined using ANCOVA, with EBV-DNA as a covariate. ANCOVA revealed a significant interaction between EBV-DNA and HHV-8 DNA but no significant interaction between thrombocytopenia and EBV-DNA. Post hoc tests revealed that HHV-8 DNA was significantly higher in subjects with platelet counts <150,000 platelets/μl than in subjects with platelet counts >150,000 platelets/μl (Fig. 7a). HHV-8 DNA was detected in 11 of 14 (78.6%) subjects with low platelet counts,

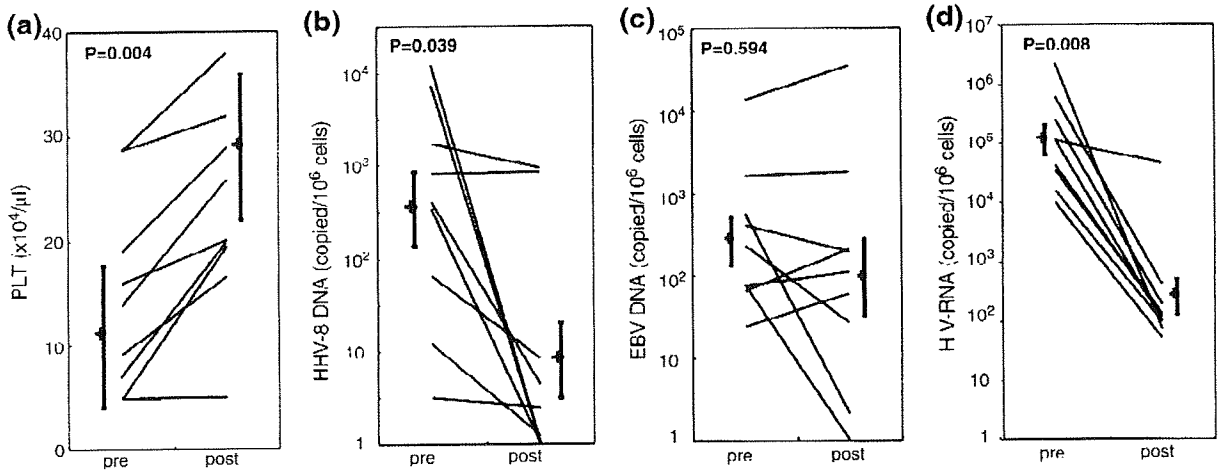

FIG. 6. Changes in the platelet counts, HHV-8 DNA loads, EBV-DNA loads, and HIV-RNA of pre- and post-ART. Platelet counts (a), HHV-8-DNA (b), EBV-DNA (c), and HIV-RNA (d) of the nine subjects were analyzed before (pre) and after 2 or 3 months of initiation of ART (post). The viral loads were $\log_{10}$ transformed. Bar shows the mean and standard deviation. p-values for the difference between the two time points were estimated by the Wilcoxon signed rank test.

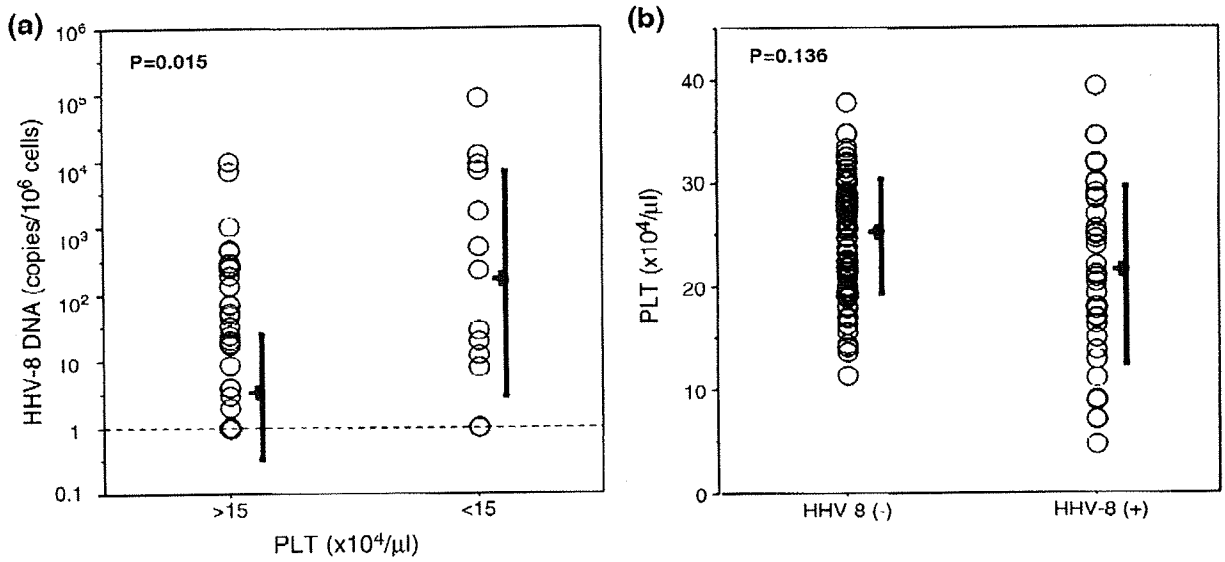

FIG. 7. Relationship between HHV-8 DNA and mild thrombocytopenia.(a) HHV-8 DNA load in leukocytes in subjects with platelet counts of <150,000 platelets/mm^3 or with platelet counts of ≥150,000 platelets/mm^3. HHV-8 copy numbers per 10^6 leukocytes are shown. The bar shows the mean and standard deviation. The *p*-values were estimated by a post hoc test after ANCOVA with EBV DNA as a covariate. (b) Platelet counts among the HIV-1-positive subjects with or without HHV-8 DNA. The bar shows the mean and standard deviation. The *p*-values were estimated by a post hoc test after ANCOVA with EBV-DNA as a covariate. The dotted line shows the detection limit of the assay.

whereas it was found in 26 of 111 (23.4%) of subjects with normal platelet counts ($p = 0.0059$; estimated by post hoc test after ANCOVA with the prevalence of EBV-DNA as a covariate). The platelet counts were lower in HHV-8 DNA-positive subjects than in HHV-8 DNA-negative subjects, *but not significanlyt* (Fig. 7b). In subjects without ART, HIV-RNA might be a confounding factor, and thus ANCOVA was conducted with HIV-RNA and EBV-DNA as covariates.

In subjects without ART, HHV-8 DNA was also significantly higher in subjects with mild thrombocytopenia with an adjustment for EBV-DNA and HIV-RNA ($p = 0.013$, data not shown). HHV-8 DNA was not detected in the 12 subjects with autoimmune thrombocytopenia. We also examined the correlation between HHV 8 DNA loads and platelet counts in subjects without KS, because the subject with KS might have occult Castleman's disease, which can cause thrombocytopenia. HHV-8 DNA was significantly higher in subjects with platelet counts of <150,000 platelets/μl than in subjects with platelet counts of ≥150,000 platelets/μl [$\log_{10}$ (HHV-8) = 2.103 ± 1.797 vs. 0.438 ± 0.924; $p = 0.0285$]. HHV-8 DNA was detected in 9 of 12 (75.0%) subjects with low platelet counts, whereas it was found in 25 of 108 (23.1%) subjects with normal platelet counts ($p = 0.0002$). The platelet counts were significantly lower in HHV-8 DNA-positive subjects than in HHV-8 DNA-negative subjects ($21.5 + 8.61/\mu$l vs. $25.0 + 5.56/\mu$l; $p = 0.1319$).

Discussion

Some previous studies have examined the prevalence of HHV-8 infection in HIV-1-infected subjects. A unique aspect of this study is that the effect of HHV-8 DNA in the peripheral blood leukocytes on HIV-1 infection was examined; a significant association between HHV-8 DNA and the clinical appearance of HIV-1 infection, especially thrombocytopenia complicated with HIV-1, was found.

Some reports have shown that the presence of KS was associated with the prevalence of HHV-8 DNA in leukocytes[14] and the severity was associated with HHV-8 DNA loads in leukocytes.[15] The current data also showed a certain association with the presence of KS and HHV-8 DNA load, but in this study there were only five subjects with KS, because the prevalence of KS in HIV-1-infected patients in Japan is low. As a result, it was impossible to determine whether the HHV-8 DNA load predicted the onset and severity of KS. To draw any conclusions concerning the clinical and prognostic utility on KS, more subjects with KS need to be analyzed.

HHV-8 encodes homologues of cytokine and cytokine response genes, such as viral interleukin-6 (vIL-6)[16] and viral interferon regulatory factor (vIRF). vIL-6 can bind the gp130 receptor to activate IL-6 response genes and promote B cell activation, which is the mechanism of hypergammaglobulinemia induced by HHV-8 infection. It was also found that HHV-8 plays an important role in hypergammaglobulinemia, which is often found in subjects with HIV-1 infection.

The current data showed that HIV-RNA load in the serum of HHV-8 DNA-positive subjects was significantly higher than that found in the serum of HHV-8 DNA-negative subjects. HHV-8 replication is activated by HIV-Tat,[17] and it has been reported that HHV-8 reactivation is associated with HIV-1 acute infection.[18] Furthermore, an open reading frame (ORF), the major transactivator of the HHV-8 lytic cycle, can also induce increased levels of HIV replication. In conclusion, these findings show that HHV-8 coinfection is associated with

immunological characteristics and disease severity of HIV infection.

ART reduced HHV-8 DNA loads as well as HIV-RNA (Fig. 4). The inhibition of HIV-1 replication with ART probably leads to the reconstitution of the immune system and regenerates effective immune responses against HHV-8. Moreover, protease inhibitors block the production of inflammatory cytokines,[19] which in turn may result in the downregulation of HHV-8 replication. In this longitudinal study during ART, HHV-8 DNA loads transiently increased in three subjects soon after the initiation of ART. This might be due to the disorder of cytokine balance, which occurs transiently in the process of the reconstitution of the immune system, and this might be one of the explanations for the immune reconstitution syndrome of KS. Some inflammatory cytokines are induced in immune reconstitution syndromes. It has recently been reported that some cytokines, which are associated with the development of KS, were elevated in other reconstitution syndromes.[20] It is possible that reactivation of HHV-8 by ART might activate the inflammation of the other reconstitution syndromes by the increase in vIL-6 or other cytokines.

The difference between HHV-8 DNA in the subjects with platelet counts of <150,000 platelets/μl and in the subjects with platelet counts of ≥150,000 platelets/μl was significant, but the difference between the platelet counts in the HHV-8 DNA-positive subjects and the platelet counts in the HHV-8 DNA-negative subjects was not significant. This is because HHV-8 DNA correlates with "mild" thrombocytopenia.

This is the first report that analyzed the association between HHV-8 DNA and thrombocytopenia in HIV-1-infected subjects, though there are a few studies that showed the relationship between HHV-8 infection and hematological disorders[21] or posttransplantation bone marrow failure in HIV-1-negative subjects.[22] Some case reports have described thrombocytopenia complicated with HHV-8-induced Castleman's disease, hemophagocytic syndrome, or other inflammatory syndromes. The HIV-1-positive subjects sometimes develop thrombocytopenia, which has been thought to be caused by an autoimmune mechanism. Recently, antibody cross-reactivity between a known epitope region of HIV-1 proteins and platelet GPIIIa19-66 has been reported to be responsible for HIV-1-associated thrombocytopenia.[23] However, the present study showed that HHV-8 also contributes independently to the thrombocytopenia complicated in HIV-1 infection.

The molecular mechanism of thrombocytopenia induced by HHV-8 remains to be established, but it is possible that some cytokines or chemokines induced by HHV-8 might be involved in thrombocytopenia. For example, interleukin-8 (IL-8), which is induced by HHV-8 infection,[24] binds the receptors on megakaryocytes and platelets and inhibits megakaryocytopoiesis.[25] Furthermore, HHV-8 is reported to infect CD34-positive hematopoietic progenitor cells[26,27]; HHV-8 harboring CD34-positive cells might also contribute to hematological disorders including thrombocytopenia.[28] Another possible explanation for thrombocytopenia is an autoimmune mechanism such as immune thrombocytopenia (ITP). Chronic stimulation of the B cell clones induced by HHV-8 could favor the production of autoantibodies, some of which might react against platelet. In fact, Evan's syndrome complicating multicentric Castleman's disease[29] and ITP complicating KS have been reported.[30]

In Italy, the area of subendemicity of HHV-8, the presence of HHV-8 DNA in HIV-1-negative elderly people has been reported to be associated with mild thrombocytopenia.[31] In Japan, HHV-8 infection in HIV-1-negative subjects is rare; as a result, when the 12 subjects with autoimmune thrombocytopenia were examined, the association between HHV-8 and autoimmune thrombocytopenia in HIV-1-negative subjects was not distinct. However, the fact that HHV-8 DNA was not detected in the HIV-1-negative subjects with autoimmune thrombocytopenia suggests that HHV-8 is not a main causative agent of autoimmune thrombocytopenia.

In conclusion, a significant association was observed between HHV-8 infection and the clinical appearance of HIV-1 infection, especially in regard to mild thrombocytopenia, hypergammaglobulinemia, and disease severity. It is possible that some of these effects of HHV-8 infection are related to other independent etiological cofactor or some other viruses. However, it may be useful to monitor HHV-8 DNA in leukocytes of HIV-1-positive subjects to assess and predict disease severity and to select the optimal treatment modalities.

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Disclosure Statement

No competing financial interests exist.

References

1. Ablashi DV, Chatlynne LG, Whitman JE Jr, and Cesarman E: Spectrum of Kaposi's sarcoma-associated herpesvirus, or human herpesvirus 8, diseases. Clin Microbiol Rev 2002; 15:439–464.
2. Miller CS, Berger JR, Mootoor Y, Avdiushko SA, Zhu H, and Kryscio RJ: High prevalence of multiple human herpesviruses in saliva from human immunodeficiency virus infected persons in the era of highly active antiretroviral therapy. J Clin Microbiol 2006;44:2409–2415.
3. Challine D, Roudot-Thoraval F, Sarah T, *et al.*: Seroprevalence of human herpes virus 8 antibody in populations at high or low risk of transfusion, graft, or sexual transmission of viruses. Transfusion 2001,41.1120–1125.
4. Stein L, Carrara H, Norman R, Alagiozoglou L, Morris L, and Sitas F: Antibodies against human herpesvirus 8 in South African renal transplant recipients and blood donors. Transpl Infect Dis 2004;6:69–73.
5. Fujii T, Taguchi H, Katano H, *et al.*: Seroprevalence of human herpesvirus 8 in human immunodeficiency virus 1-positive and human immunodeficiency virus 1-negative populations in Japan. J Med Virol 1999;57:159–162.
6. Katano H, Iwasaki T, Baba N, *et al.*: Identification of antigenic proteins encoded by human herpesvirus 8 and seroprevalence in the general population and among patients with and without Kaposi's sarcoma. J Virol 2000;74:3478–3485.

7. Mesri EA, Cesarman E, Arvanitakis L, *et al.*: Human herpesvirus-8/Kaposi's sarcoma-associated herpesvirus is a new transmissible virus that infects B cells. J Exp Med 1996;183:2385–2390.
8. Sirianni MC, Vincenzi L, Topino S, *et al.*: Human herpesvirus 8 DNA sequences in CD8 – T cells. J Infect Dis 1997;176:541.
9. Sirianni MC, Uccini S, Angeloni A, Faggioni A, Cottoni F, and Ensoli B: Circulating spindle cells: Correlation with human herpesvirus 8 (HHV 8) infection and Kaposi's sarcoma. Lancet 1997;349:255.
10. Minami R and Yamamoto M: A case of HIV-1 and HHV-8-associated Castleman disease with a relapsing high fever and lymphoadenopathy. Kansenshogaku Zasshi 2006;80: 423–427.
11. Polstra AM, Van Den Burg R, Goudsmit J, and Cornelissen M: Human herpesvirus 8 load in matched serum and plasma samples of patients with AIDS-associated Kaposi's sarcoma. J Clin Microbiol 2003;41:5488–5491.
12. Kimura H, Morita M, Yabuta Y, *et al.*: Quantitative analysis of Epstein-Barr virus load by using a real-time PCR assay. J Clin Microbiol 1999;37:132–136.
13. Nagai H, Wada K, Morishita T, Utsumi M, Nishiyama Y, and Kaneda T: New estimation method for highly sensitive quantitation of human immunodeficiency virus type 1 DNA and its application. J Virol Methods 2005;124:157–166.
14. Boivin G, Gaudreau A, Toma E, *et al.*: Human herpesvirus 8 DNA load in leukocytes of human immunodeficiency virus-infected subjects: Correlation with the presence of Kaposi's sarcoma and response to anticytomegalovirus therapy. Antimicrob Agents Chemother 1999;43:377–380.
15. Laney AS, Cannon MJ, Jaffe HW, *et al.*: Human herpesvirus 8 presence and viral load are associated with the progression of AIDS-associated Kaposi's sarcoma. AIDS 2007;21:1541–1545.
16. Aoki Y, Jones KD, and Tosato G: Kaposi's sarcoma-associated herpesvirus-encoded interleukin-6. J Hematother Stem Cell Res 2000;9:137–145.
17. Harrington W Jr, Sieczkowski L, Sosa C, *et al.*: Activation of HHV-8 by HIV-1 tat. Lancet 1997;349:774–775.
18. Lennette ET, Busch MP, Hecht FM, and Levy JA: Potential herpesvirus interaction during HIV type 1 primary infection. AIDS Res Hum Retroviruses 2005;21:869–875.
19. Wasmuth JC, Nischalke HD, Jütte A, *et al.*: Chemokine mRNA levels in mononucleated cells of HIV-infected patients before and after initiation of PI- versus NNRTI-containing HAART. Antiviral Res 2004;61:207–212.
20. Tamburini J, Grimaldi D, Chiche JD, Bricaire F, and Bossi P: Cytokine pattern in Kaposi's sarcoma associated with immune restoration disease in HIV and tuberculosis co-infected patients. AIDS 2007;21:1980–1983.
21. Tsai WH, Lee YM, Ing-Tiau Kuo B, *et al.*: Increased seroprevalence of human herpesvirus 8 in patients with hematological disorders. Acta Haematol 2005;114:95–98.
22. Cuzzola M, Irrera G, Iacopino O, *et al.*: Bone marrow failure associated with herpesvirus 8 infection in a patient undergoing autologous peripheral blood stem cell transplantation. Clin Infect Dis 2003;37:102–106.
23. Li Z, Nardi MA, and Karpatkin S: Role of molecular mimicry to HIV-1 peptides in HIV-1-related immunologic thrombocytopenia. Blood 2005;106:572–576.
24. Sun Q, Matta H, Lu G, and Chaudhary PM: Induction of IL-8 expression by human herpesvirus 8 encoded vFLIP K13 via NF-kappaB activation. Oncogene 2006;25:2717–2726.
25. Kowalska MA, Ratajczak J, Hoxie J, Brass LF, Gewirtz A, Poncz M, and Ratajczak MZ: Megakaryocyte precursors, megakaryocytes and platelets express the HIV co-receptor CXCR4 on their surface: Determination of response to stromal-derived factor-1 by megakaryocytes and platelets. Br J Haematol 1999;104:220–229.
26. Henry M, Uthman A, Geusau A, *et al.*: Infection of circulating CD34 + cells by HHV-8 in patients with Kaposi's sarcoma. J Invest Dermatol 1999;113:613–616.
27. Wu W, Vieira J, Fiore N, *et al.*: KSHV/HHV-8 infection of human hematopoietic progenitor (CD34+) cells: Persistence of infection during hematopoiesis *in vitro* and *in vivo*. Blood 2006;108:141–151.
28. Quinn JP, Gilligan OM, and Horgan M: Evan's syndrome complicating multicentric Castleman's disease—dramatic response to rituximab. Eur J Haematol 2004;73:384–385.
29. Brown EE, Whitby D, Vitale F, *et al.*: Correlates of human herpesvirus-8 DNA detection among adults in Italy without Kaposi sarcoma. Int J Epidemiol 2005,34:1110–1117.
30. Muela Molinero A, Ballesteros del Rio B, Sandoval Guerra V, and Llor Banos J: Evans syndrome as presentation of multicenter Castleman disease. Rev Clin Esp 2003;203:616–617.
31. Toyohama T, Nagasaki A, Miyagi J, *et al.*: Kaposi's sarcoma in a human immunodeficiency virus negative patient treated with corticosteroid for idiopathic thrombocytopenic purpura. Intern Med 2003;42:448–449.

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