The Journal of AIDS Research Vol. 11 No. 3 2009

例 #4, #7 に関しては，試験終了後も観察を継続しており，HIV-RNA 量の変動により，BID への変更も検討している。

試験期間に発現した有害事象はいずれも軽微であり，嘔気，胃部不快感などの消化器症状は服薬後数時間で発現し，時間経過とともに消失する例が多かった。これは，QD への変更で服用後の血中濃度が BID に比べ上昇したことによって発現した症状であると考えられた。また，これらの症状が時間経過と共に消失したことから，血中濃度の上昇による副作用は，時間経過とともに軽減できる症状であったと考えられた。予想された下痢の頻度が海外報告に比べ少なかったことから，今回の試験における QD の忍容性を確認することができた。本試験の対象となった患者は，すでに LPV/r を BID で服用した経験を有していたことが，下痢の頻度の少なかった要因の一つであると推測された。

本試験を実施した結果，QD は BID と同等のウイルス抑制効果を持ち，CD4 細胞数の維持が可能であることが確認され，試験期間中に脂質代謝パラメータの変動，重篤な有害事象が認められなかったことから，24 週間における安全性と有効性を確認することができた。日本では現在 QD の適応はないものの，今回の結果が日本における QD 投与の有用性確認の第一歩となったものと考える。

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Results of Switching Lopinavir/Ritonavir from Twice Daily to Once Daily Dosing

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Objectives : We examined the efficacy and safety of switching patients with lopinavir (LPV) trough concentrations of 6.00μg/mL or more on twice daily (bid) lopinavir/ritonavir (LPV/r) dosing to once daily.

Subjects and Methods : Eight patients at our hospital treated with LPV/r bid with LPV trough concentrations over 6.00μg/mL were enrolled in this study. Adverse events, trough concentrations, HIV-RNA loads, CD4 cell counts, total cholesterol, high-density lipoprotein (HDL) cholesterol, and triglycerides were examined 4, 8, 12, 16, 20, and 24 weeks after switching to once daily dosing and compared to the levels during bid dosing.

Results : The LPV trough concentration of the eight enrolled patients before the switch was 10.99±2.75μg/mL (mean±SD, range 7.46–5.85). Although the mean LPV trough concentration declined to 2.28±1.72μg/mL (range 0.41–5.85) 4 weeks after the switch, the HIV-RNA load remained undetectable for all subjects throughout the 24-week study. No changes in CD4 cell counts, total cholesterol, HDL cholesterol, or triglycerides were observed. Gastrointestinal symptoms, such as constipation and nausea, emerged as new adverse events, but the increase in diarrhea was minimal.

Conclusions : Switching patients with relatively high LPV blood concentrations and a stable clinical course to once daily dosing was confirmed to be safe and effective for 24 weeks. We consider the results to be the first step in confirming the efficacy of once daily dosing in Japan.

Key words : HAART, lopinavir, ritonavir, pharmacokinetics, once daily

 The Journal of AIDS Research

研究ノート

硫酸アタザナビルの血中濃度が高値の患者を対象とした，ATV/rからATV$_{400}$へのスイッチ臨床試験結果

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目的：ATV/r服用患者を対象に，ATVの血中濃度が高値を示す患者をATV$_{400}$へ変更することにより，有効性を維持しつつ，副作用の軽減を図ることを目的に臨床試験を実施した。

対象および方法：2004年6月から2008年5月までの期間，当院でATV/rを服薬し，ATVのトラフ血中濃度が1.50μg/mL以上の患者5例を対象とした。ATV$_{400}$へ変更後4，8，12，16，20，24週目にトラフ濃度，HIV-RNA量，T-Bil，T-Cho，TGを測定し，Dunnettの多重分析法を用いて比較検討した。

結果：対象患者5例のATVトラフ濃度（mean±S.D.）は，1.95±0.39μg/mL（range：1.54-2.56）であった。変更後4週目ATVトラフ濃度の平均値は0.26±0.18μg/mL（range：0.10-0.52）に低下したが，HIV-RNA量は臨床試験を実施した24週間を通じて，全例感度未満を持続した。変更前後で，T-Bil，T-Choは有意に低下した（$p<0.05$）。

結論：ATVトラフ濃度が1.50μg/mL以上を示す患者がATV$_{400}$へ変更した場合の24週間における安全性と有効性が示唆された。T-BilとT-Choが変更後に有意に低下したことは，黄疸の軽減と患者のQOLの向上につながり，長期服用による副作用発現を未然に防止できたものと考えられた。

キーワード：HAART，ATV，RTV，血中濃度，Bil

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緒　言

硫酸アタザナビル（ATV）は，米国で2003年6月にHIV感染症の治療薬として承認されたプロテアーゼ阻害薬（PI）であり，1日1回投与が可能なPIとして，米国DHHS（Department of Health and Human Services）のガイドラインでは第一選択薬として推奨されている[1]。ATVのヒトにおける主な代謝経路は，一酸化及び二酸化反応であり，ヒト肝ミクロゾームを用いたin vitro試験から，チトクロームP450の分子種CYP3A4により代謝を受けることが確認されている[2]。ATVと併用するリトナビル（RTV）は，CYP3A4を強力に阻害することが知られており，少量のRTVを併用することによりPIの効果を増強するブースト療法が一般的となっている[3]。ブーストしないATV 400 mg投与（ATV$_{400}$）は，RTV 100 mgでブーストしたATV 300 mg投与（ATV/r）に比べ，トラフレベルの血中濃度（トラフ濃度）が87%，AUCが51%低下することが報告されている[2]。ATV/rでは症例によってATVの血中濃度の上昇が，Total Bilirubin（T-Bil）の上昇を惹起している可能性が示唆される。RTVは冷蔵保存が必要であることから，患者の利便性に支障があり，RTVの相互作用上の併用注意薬も多く，併用を避けざるを得ない症例も経験する。今回，ATV/r服用患者においてATVのトラフ濃度が高値を示す患者に対し，ATV$_{400}$へ変更することにより，有効性を維持しつつ，副作用の軽減を図ることを目的に臨床試験を実施した。なお本研究は，国立病院機構大阪医療センターの倫理委員会に相当する受託研究審査委員会の承認を得た（承認番号：0710）。

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対象および方法

国立病院機構大阪医療センター免疫感染症科に通院し，少なくとも14日間以上，ATV/rを含むHAARTを施行し，ATVのトラフ濃度が1.50μg/mL以上の患者で，問診により血中濃度測定前1週間の服薬率が100%と見込まれた患者に対し本研究の趣旨の説明を行い，試験参加の同意を文書で得た5例を対象とした。併用した核酸系逆転写酵素阻害薬（NRTI）は，全例，アバカビル/ラミブジン合剤（EZC）であった。同意取得後，ATV/rからATV$_{400}$へ変更

The Journal of AIDS Research Vol. 11 No. 1 2009

を行った。対象となった患者の平均年齢（mean±S.D.）は46±12歳（33-63歳）で，すべて男性であった。

なお，テノホビル（TDF）並びにTDF配合剤，プロトンポンプ阻害剤（PPI），高脂血症剤等，ATV血中濃度に影響を及ぼすと考えられる薬剤の投与を受けている患者は本臨床試験の対象外とした。調査期間は2004年6月1日から2008年5月31日までとした。

血中濃度測定は以下の方法で行った。ヘパリンナトリウムを添加した試験管に，1回5 mLの血液を採取し，10℃以下3000回転10分間遠心分離し，ポリプロピレン製のスクリューキャップ付きチューブに血漿を2 mL分注し，分析開始まで－80℃で凍結保存した。測定はHPLC法を用い，株式会社積水メディカルで実施した。また，血中濃度測定は，厚生労働科学研究費補助金「抗HIV薬の血中濃度に関する臨床研究」により実施した。

ATV_{400}へ変更後4，8，12，16，20，24週目に採血を実施し，ATVのトラフ濃度，HIV-RNA量，T-Bil，T-Cho，TGを測定し，ATV_{400}への変更前後の24週間について比較検討を行った。T-Bil，T-Cho，TGについては，変更前後の両群間の変動（%）をDunnettの多重分析法を用いて解析した。

結　果

対象患者の治療変更前後の血中濃度を表1，臨床検査値を表2に示す。ATVトラフ濃度（mean±S.D.）は1.95±0.39μg/mL（range：1.54-2.56）であった。ATV_{400}への変更後4週目に，患者のATV血中濃度は0.26±0.18 μg/mL（range：0.10-0.52）に低下し，その後24週までほぼ一定の値を示した。HIV-RNA量は臨床試験を実施した24週間を通じて，全例感度未満を持続していた。変更後のT-Bilの変動（%）において4，20，24週に変更前に比べ有意な低下が認められた（$p<0.05$）。また，T-Choの変動（%）は12，16，20，24週に有意な低下が認められた（$p<0.05$）。TGについては変更後4週以降，16週を除き，有意差は認められないものの低下傾向を示した。

考　察

PIのウイルス学的効果は血中濃度と相関することから[4,5]，ウイルス学的有効性を考えれば，より高く血中濃度を維持することが出来るATV/rの有効性はATV_{400}より勝っていると考えられる。しかし，ATVのトラフ濃度が高値となるとT-Bilの上昇を惹起することが複数報告されている[6,7]。また，ATVによるT-Bilの上昇は，一般に肝機能障害とは関係なく[8]，安全性に問題はないとされているが，黄疸という副作用症状は患者の外観の変化を来すことから，その発現する症状を嫌い，服薬変更を希望する場合や，患者によっては服薬の自己中断を行った症例も経験している。ATVの血中濃度は，有効かつ黄疸等の副作用が発現しない濃度でコントロールすることが最も望ましい。ブースタとして使用するRTVは冷蔵庫での保存が必要であり，患者にとっては利便性を阻害する要因の一つとなり，さらにRTVは相互作用のある薬剤も多いため，併用を避けざ

表1　ATV trough plasma concentration (μg/mL)

patient	Baseline	week 4	week 8	week 12	week 16	week 20	week 24
1	1.54	0.10	0.10	0.16	0.15	0.17	0.41
2	1.72	0.23	0.16	0.15	0.16	0.16	0.13
3	2.03	0.35	0.23	0.29	0.24	0.31	0.15
4	1.90	0.11	0.42	0.20	0.28	0.10	0.10
5	2.56	0.52	0.59	0.20	0.36	0.69	0.35
Mean (S.D.)	1.95 (0.39)	0.26 (0.18)	0.30 (0.20)	0.20 (0.06)	0.24 (0.09)	0.29 (0.24)	0.23 (0.14)

表2　Laboratory parameters

Parameter		n	Baseline	week 4	Week 8	week 12	week 16	week 20	week 24
T-Bil	(mg/dL)	5	3.46 (1.45)	1.56 (0.50)*	2.14 (0.58)	2.04 (0.63)	1.86 (0.57)	1.43 (0.87)*	1.72 (0.29)*
T-Cho	(mg/dL)	5	205.6 (41.2)	194.4 (45.9)	185.2 (30.7)	172.6 (29.8)*	180.8 (39.6)*	187.5 (25.2)*	166.0 (35.0)*
TG	(mg/dL)	5	259.4 (70.4)	122.2 (44.9)	134.2 (41.7)	128.8 (71.0)	228.0 (103.8)	128.8 (62.3)	120.2 (49.3)
HIV-RNA	(copies/mL)	5	<50	<50	<50	<50	<50	<50	<50

Data expressed as Mean (S.D.)

* Significant difference from the baseline at $p<0.05$ using parametric Dunnett's multiple comarison test.

るを得ない場合もある。従って，治療の有効性を高めるためには高い血中濃度を維持することは効果的であるが，高い血中濃度を示す患者に対し，ブースタであるRTVの使用を中止し，ATV 400 mg投与に変更し，その有用性を検討することは臨床的意義があると考える。

今回我々は，ATV/r服用患者においてATVのトラフ濃度が1.50 μg/mL以上を示す患者に焦点を絞り，ATV_{400}へ変更することで，有効性の維持と，副作用の軽減について検討した。対象患者のトラフ濃度を1.50 μg/mL以上にした設定根拠は，ATV/rを服用した健常成人が，ブーストしないATV_{400}へ変更した場合，トラフ濃度の幾何平均値が1.23 μg/mLから0.16 μg/mLへ87%，AUCが57.0 μg・h/mLから28.1 μg・h/mLへ51%低下する報告に基づき[2]，DHHSのガイドラインが推奨するATV目標トラフ濃度0.15 μg/mL[1]を維持するためには，トラフ濃度が1.50 μg/mL以上必要であると推定した。

ATV/rからATV_{400}へ変更後，トラフ濃度の平均値は1.95±0.39 μg/mLから4週後には0.26±0.18 μg/mLまで約86%低下した。変更後の低下率は先の報告[2]と類似しており，トラフ濃度の平均値は24週間において0.15 μg/mL以上を維持したことから，今回対象患者のトラフ濃度を1.50 μg/mL以上にした設定根拠は，妥当であると考える。しかし，表1に示すように，測定した患者の血中濃度には目標トラフ濃度を下回ったデータもあり，臨床試験を実施した24週間における患者のHIV-RNA量は，全例検出限界未満を維持していたものの，長期の安全性を検討するためのATV/r服用患者におけるATVトラフ濃度の設定に関しては検討が必要と考える。

ATV_{400}へ変更後，T-Bilが有意に低下したことは，黄疸の軽減と患者のQOLの向上につながった。HAARTによる高脂血症は，主にPIが関与しており，服用期間が長いほど虚血性心疾患の頻度が増加し，1年のHAARTへの暴露で年間発生率が26%増加することが報告されている[9,10]ことから，T-Cho，TGが変更後に低下したことは，長期服用による副作用発現を未然に防止できたものと考えられた。ATVのトラフ濃度が高値を示す患者に対し，ATV_{400}へ変更した24週間における安全性と有効性が示唆された。今後，ATV/rからATV_{400}への変更について長期間の安全性と有効性を検討するためには，より多くの症例について長期間観察を行う臨床試験が必要である。

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The Journal of AIDS Research Vol. 11 No. 1 2009

Successful Switch from ATV/r to ATV 400 in HIV-infected Patients with High Plasma ATV Concentrations

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Objectives : We examined the changeover to atazanavir 400 mg (ATV 400) in patients taking atazanavir/ritonavir (ATV/r) with high plasma ATV concentrations, with the goal of reducing adverse effects, while maintaining efficacy.

Subjects and Methods : The subjects were five patients taking ATV/r at our hospital between June 2004 and May 2008 who had ATV trough plasma concentrations of 1.50μg/mL or more. The trough plasma concentration, HIV-RNA level, total bilirubin (T-Bil), total cholesterol (T-Cho), and triglycerides (TG) were measured 4, 8, 12, 16, 20, and 24 weeks after switching to ATV 400 and the results were compared using Dunnett's multiple analysis.

Results : The ATV trough concentration of the five patients averaged 1.95$\pm$0.39μg/mL (mean$\pm$S.D. ; range 1.54 to 2.56μg/mL). Four weeks after the switchover, it had declined to 0.26$\pm$0.18μg/mL (range 0.10 to 0.52μg/mL), while the HIV-RNA levels in all cases were maintained below the detection level of 50 copies/mL at 24 weeks. After the switchover, T-Bil and T-Cho declined significantly ($p<0.05$).

Conclusions : It appears safe and effective to switch patients with ATV trough concentrations of 1.50μg/mL and above to ATV 400. The significant declines in T-Bil and T-Cho after the switchover were linked to the decline in jaundice and improved quality of life (QOL) of the patients. It was thought that the adverse effects caused by taking ATV/r for a prolonged period were prevented.

Key words : HAART, atazanavir, ritonavir, pharmacokinetics, bilirubin

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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 ($ß_2M$) 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′-CGGAAGCCCTCTGGA CTTC-3′, and reverse, 5′-CCCTGTTTATCCGATGGAATG – 3′, and probe 5′-FAM-TGTACACGCACGAGAAATGCGCC-TAMRA-3′;[12] ß $_2$M forward, 5′-CAGCAAGGACTGGTCTTT CTATCTCT-3′, and reverse, 5′-ACCCCACTTAACTATCTT GG-3′, and probe 5′-FAM-CACTGAAAAAGATGAGTATG CCTGCCGTGT-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 $ß_2M$ gene, since two $ß_2M$ 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.

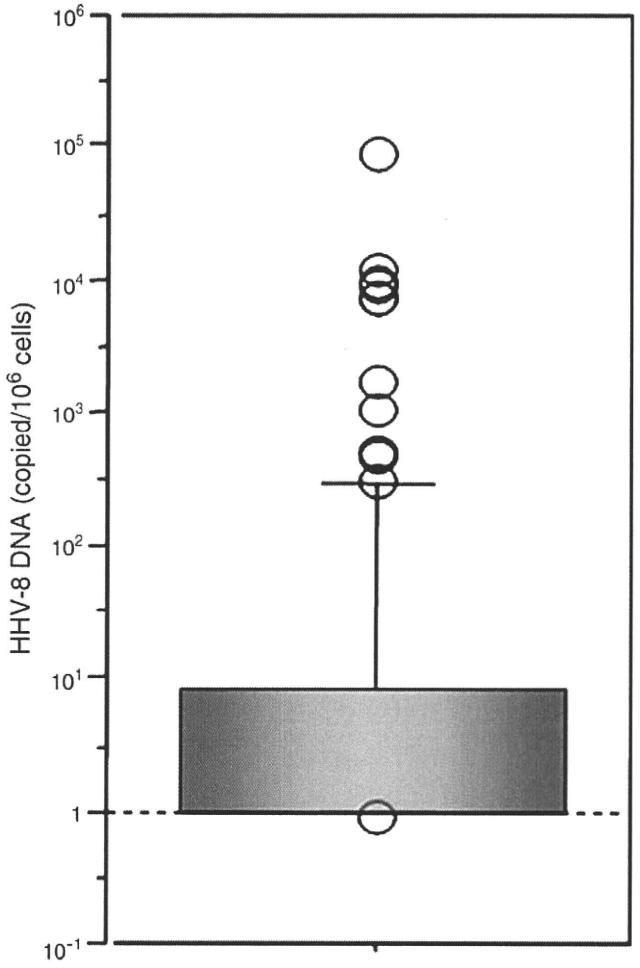

FIG. 1. Quantification of HHV-8 DNA by real-time PCR. Log_{10}-transformed HHV-8 copy numbers per 10^6 leukocytes are shown. The bar shows the mean and standard deviations. The dotted line shows the detection limit of the assay.

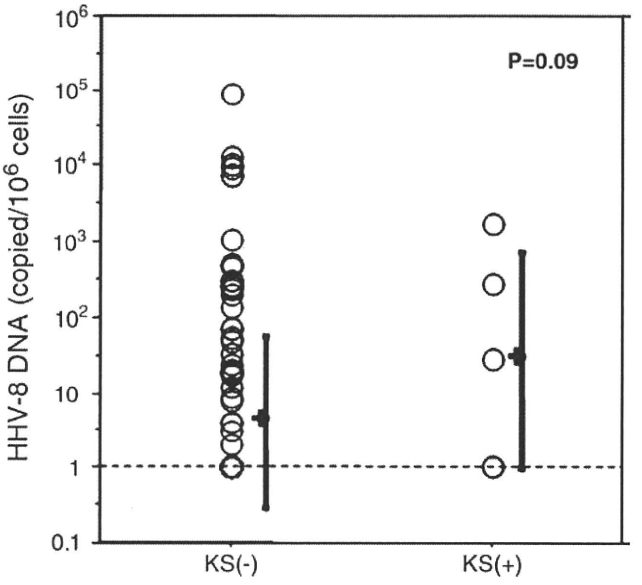

FIG. 2. HHV-8 DNA loads among the HIV-1-positive subjects with or without KS. Log_{10}-transformed HHV-8 copy numbers per 10^6 leukocytes are shown. The bar shows the mean and standard deviations. *p*-values were estimated by the Mann–Whitney *U* test. The dotted line shows the detection limit of the 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. 3.575 ± 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

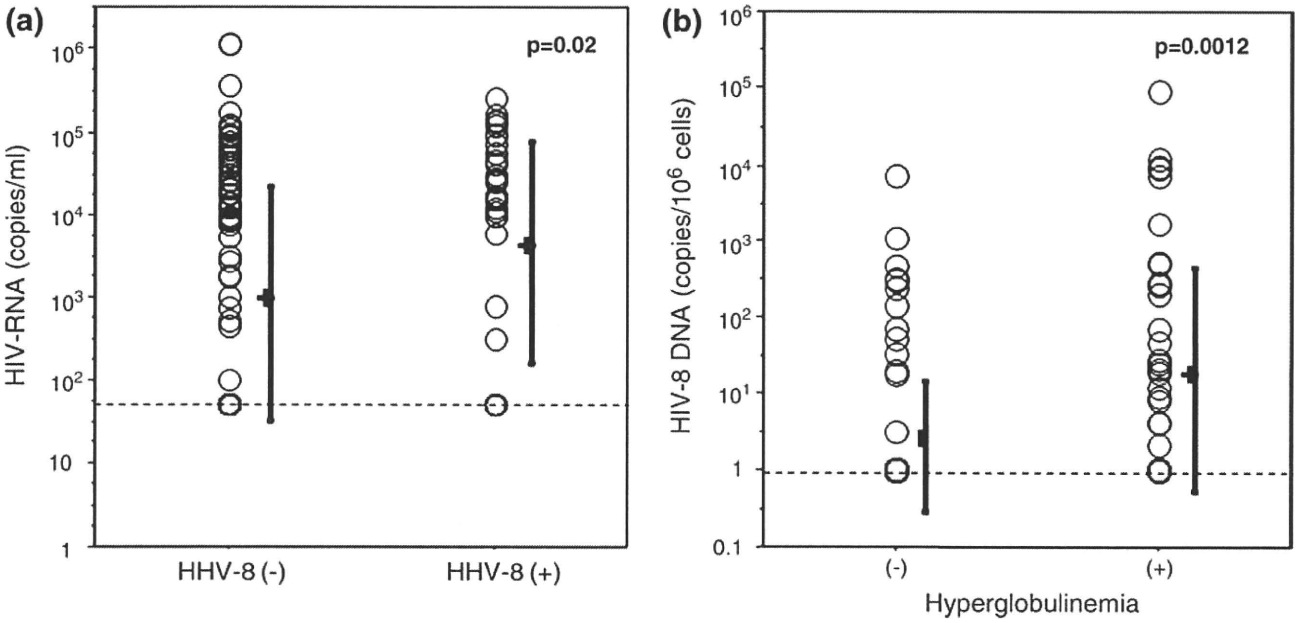

FIG. 3. The correlation between HHV-8 DNA and HIV-1-related immunovirological parameters. (**a**) HIV-RNA loads among the HIV-1-positive subjects with or without HHV-8 DNA. Log_{10}-transformed HIV-1 copy numbers/ml of serum are shown. The bar shows the mean and standard deviation. *p*-values were estimated 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

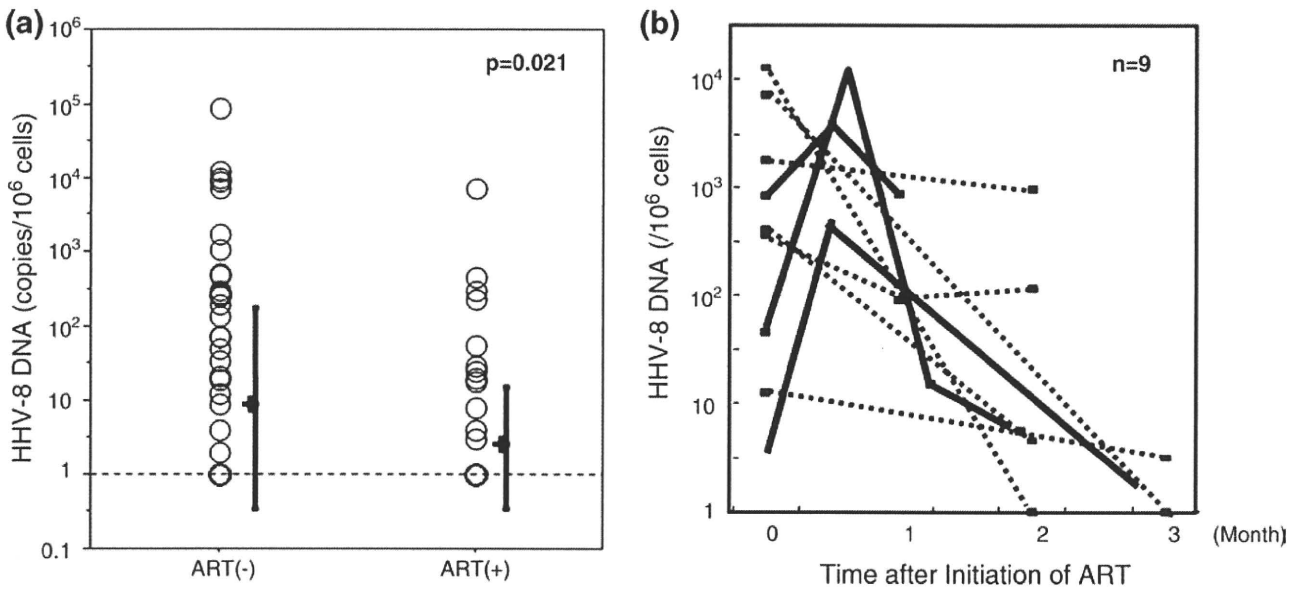

FIG. 4. The effect of ART on HHV-8 DNA. (**a**) HHV-8 DNA load in PBMCs in subjects with or without ART. HHV-8 copy numbers per 10^6 leukocytes are shown. The bar shows the mean and standard deviation. *p*-values were estimated by the Mann–Whitney *U* test. (**b**) HHV-8 DNA load after initiation of 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.

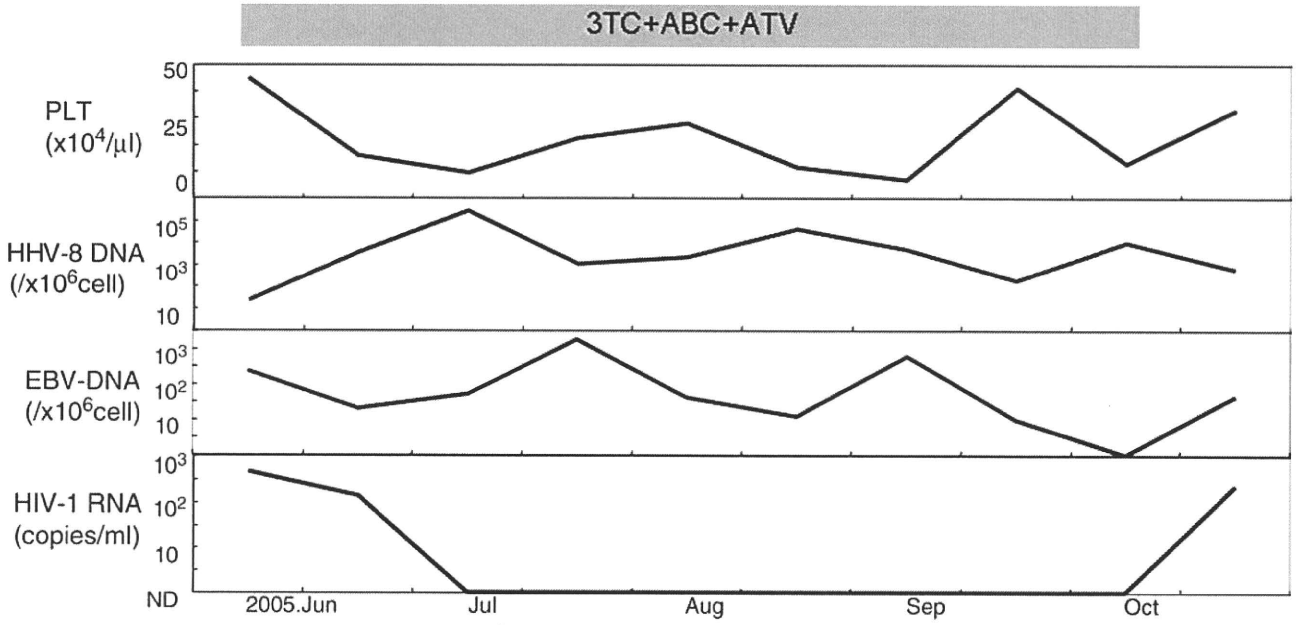

FIG. 5. Time course of the case of Castleman's disease with HIV-1 infection. Platelet counts, $\log_{10}$-transformed HHV-8 and EBV copy numbers per 10^6 leukocytes, and $\log_{10}$-transformed HIV-1 copy numbers/ml of serum are shown. The data were excerpted from Minami and Yamamoto.[10] PLT, platelet;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

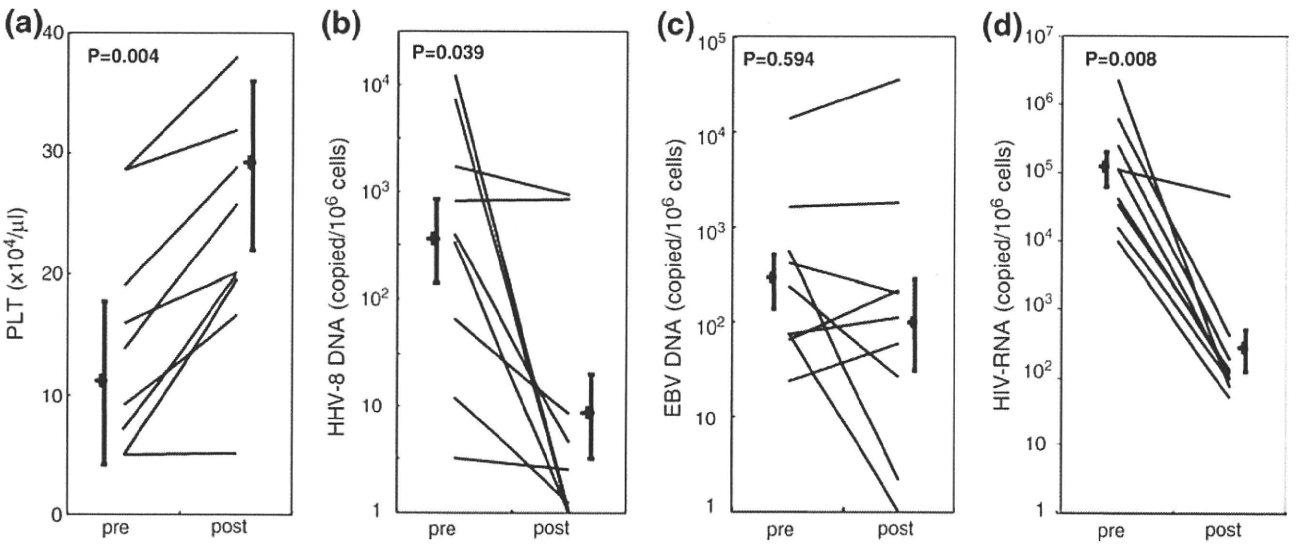

FIG. 6. Changes in the platelet counts, HHV-8 DNA loads, EBV-DNA loads, and HIV-RNA of pre- and post-ART. Platelet counts (**a**), HHV-8-DNA (**b**), EBV-DNA (**c**), and HIV-RNA (**d**) of the nine subjects were analyzed before (pre) and after 2 or 3 months of initiation of ART (post). The viral loads 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.

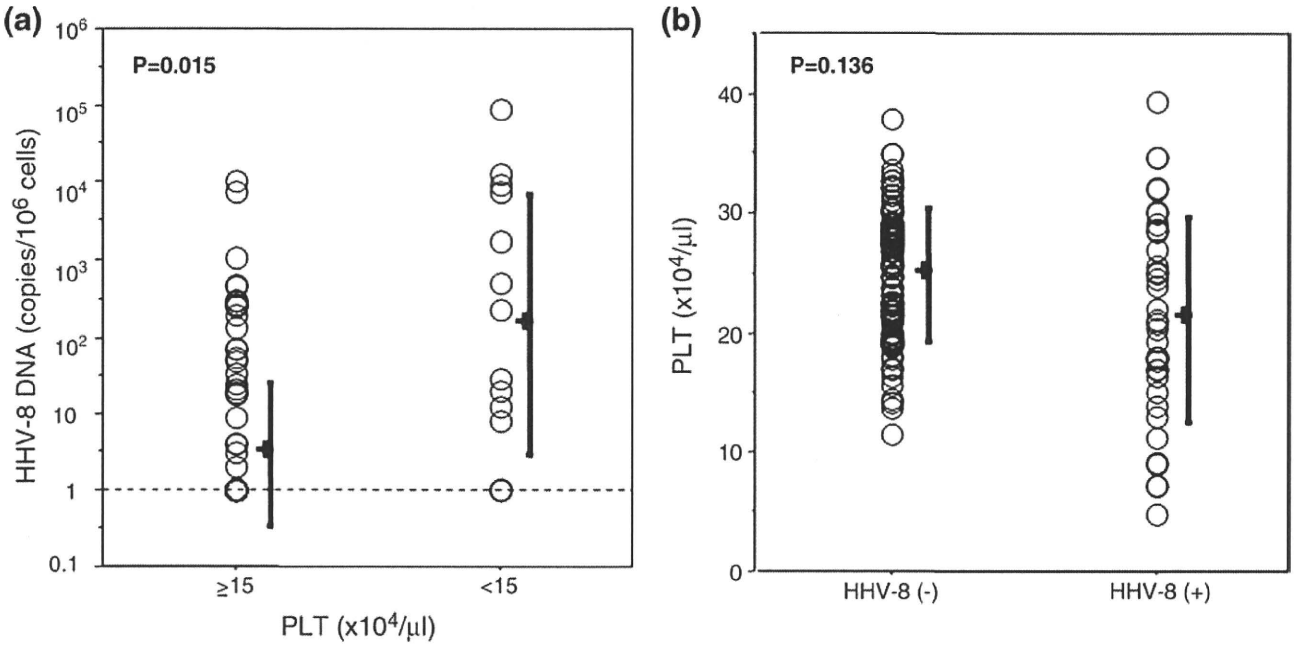

FIG. 7. Relationship between HHV-8 DNA and mild thrombocytopenia.(**a**) HHV-8 DNA load in leukocytes in subjects with platelet counts of <150,000 platelets/mm^3 or with platelet counts of ≥150,000 platelets/mm^3. HHV-8 copy numbers per 10^6 leukocytes are shown. The bar shows the mean and standard deviation. 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 ($21.5 \pm 8.61/\mu$l vs. $25.0 \pm 5.56/\mu$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 GPIIIa49-66 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.

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INTERNAL MEDICINE

ORIGINAL ARTICLE

High Molecular Weight Form of Adiponectin in Antiretroviral Drug-induced Dyslipidemia in HIV-Infected Japanese Individuals Based on *in vivo* and *in vitro* Analyses

Rumi Minami, Masahiro Yamamoto, Soichiro Takahama, Hitoshi Ando, Tomoya Miyamura and Eiichi Suematsu

Abstract

Objective High molecular weight (HMW)-adiponectin has been found to be a better negative regulator of insulin resistance than total adiponectin. The aim of this study was to investigate the influence of HMW-adiponectin on antiretroviral therapy (ART)-induced dyslipidemia in Japanese human immunodeficiency virus (HIV)-infected individuals. We also examined the effect of some antiretroviral drugs (ARVs) on adipocytes *in vitro*.

Patients and Methods Fifty-seven HIV-infected patients were enrolled in four clinical groups; (I) patients who started ART containing efavirenz (EFV); (II) patients who started ART containing a protease inhibitor without atazanavir (ATV); (III) patients who started ART containing ATV; (IV) patients who switched from ART without ATV into ART containing ATV. We measured the serum HMW-adiponectin before and one year after starting or changing ART, using an enzyme-linked immunoSorbent assay (ELISA). Furthermore, we treated the mouse adipocytes (3T3-L1) with some ARVs. The lipid content was assessed using Oil Red O staining. The expression of adiponectin was measured by quantitative real-time PCR.

Results The serum HMW-adiponectin decreased significantly in groups (I) and (II) after starting ART, and increased significantly in group (IV) after changing from ART without ATV to ART with ART. EFV, ritonavir (RTV) and nelfinavir (NFV) inhibited the expression of adiponectin mRNA in mature 3T3-L1 and to a greater extent in pre-mature 3T3-L1. This phenomenon was reversible when ARV was changed to ATV.

Conclusion Effects of the ARVs on adiponectin may vary depending on the administration of different drugs. These data suggest that the distinct metabolic effects of ARV could therefore be a consequence of their differential effects on the production of adiponectin.

Key words: HMV-adiponectin, antiretroviral therapy, dyslipidemia

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Introduction

Adiponectin is an adipokine secreted exclusively by adipocytes: this protein plays an important role in the control of systematic lipid metabolism and insulin sensitivity (1). Adiponectin forms multimers and is present in the serum as a trimer, hexamer, or as a high molecular weight (HMW) form (2). The HMW isoform most avidly binds to its receptors and stimulates AMP-activated protein kinase, one of the key molecules mediating the metabolic actions of adiponectin.

Antiretroviral therapy (ART) has prolonged survival in human immunodeficiency virus (HIV)-infected individuals, but most individuals receiving ART develop metabolic abnormalities, which include dyslipidemia (elevated plasma triglycerides and cholesterol), increased visceral and dorsocervical adipose tissue and peripheral lipoatrophy (3). Although

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HIV infection itself (4) and nucleoside reverse transcriptase inhibitors (NRTI) (5-7) have been associated with metabolic abnormalities, there are increasing clinical and epidemiological data that suggest a central role for HIV protease inhibitors (PIs) in the causation of metabolic complications (8) and atazanavir (ATV) use has been associated with a decrease in hyperlipidemia, less insulin resistance and reversal of lipodystrophy (9).

Several recent clinical studies suggest that hypoadiponectinemia might play an important role in the causation of metabolic abnormalities associated with HIV infected individuals treated with PI-based ART, especially for those with lipodystrophy (8, 10, 11), but to date, limited data have been published whether this hypoadiponectimia is due to a disturbance of adipocyte differentiation, or to the decrease in the number of adipose cells number, or a dysfunction of adipocytes. In addition, there are still no data regarding the influence of antiretroviral drugs (ARVs) on serum HMW-adiponectin levels in Japanese, about 40% of whom have a genetic variation in the adiponectin gene associated with a reduced adiponectin level (12).

This study compared the effect on HMW-adiponectin level between ATV, PIs (except for ATV), ATV replacement therapy and non-nucleoside reverse transcriptase inhibitors (NNRTI), efavirenz (EFV) in HIV-infected Japanese subjects. In addition, the influence of each of the ARVs on adipocyte development and the expression of adiponectin were evaluated, using in vitro models.

Materials and Methods

Study population

The subjects evaluated in this study consisted of 57 HIV-positive patients admitted to National Hospital Organization Kyushu Medical Center, who started ART or were changing the ART combination. The present study was conducted in accordance with the regulations of the institutional ethics committee. These individuals were enrolled into four clinical groups; (I) patients who started ART containing EFV; (II) patients who started ART containing PIs without ATV; (III) patients who started ART containing ATV; (IV) patients who replaced the ART without ATV into ART containing ATV. The Body Mass Index (BMI), serum triglyceride, low density lipoprotein (LDL) cholesterol, high density lipoprotein (HDL) cholesterol, HMW adiponectin were measured before and one year after starting or changing ART. HMW-adiponectin was measured by enzyme-linked immunosorbent Assay (ELISA), using the Human Adiponectin ELISA kit for Total and Multimers (Daiichi Pure Chemicals Co., Tokyo, Japan). Lipodystrophy was determined by a standardized, lipodystrophy-specific physical examination which recorded lipoatrophy and/or diffuse fat accumulation in the face, neck, dorsocervical spine, arms, breasts, abdomen, buttocks and legs.

Cells

The 3T3-L1 cells were purchased from the Japanese Collection of Research Bioresources. The cells were maintained in DMEM supplemented with 10% fetal bovine serum. For differentiation, post confluent cells were induced by incubation with 0.5 mM 3-isobutyl-methylxanthine and 1 μM dexamethasone for 2 days. This is followed by incubation with 10 μg/mL insulin for 2days. The cells were then maintained in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) for another 2 days. To investigate the effects of ARVs on adipogenesis, and to investigate the difference between the effect of ARVs on preadipocytes and the effect on mature adipocytes, several ARVs were added to the medium before and after differentiation of 3T3-L1 cells.

Antiretroviral drugs

Efavirenz (EFV), ritonavir (RTV), nelfinavir (NFV) were purchased from Toronto Research Chemicals Inc. (Ontario, Canada). ATV was provided by Bristol-Myers Squibb Company (NY, USA). Drug stocks in dimethyl sulfoxide (DMSO) were stored at -20°C and diluted into culture media. Vehicle control incubations received the same final DMSO concentration as all drug-treated incubations (0.1%). The IC50 /IC95 and the Cmax of each drug are as follows; EFV (IC95=1.7-25nM, Cmax= 14.2-28.8 μM), RTV (IC50= 65-289nM, Cmax= 0.84-21.9 μM), NFV (IC50=30-60nM, Cmax= 5.0-8.6 μM), and ATV (IC50=2-5nM, Cmax=4.96-8.38 μM). The cells were treated with 20 μM of EFV, RTV and ATV and 10 μM of NFV.

Quantitative real time RT-PCR

Total cellular RNA was isolated from 3T3-L1 cells, using QIAamp RNA Blood Mini (QIAGEN, Tokyo, Japan), including treatment with DNase. cDNA was generated from the RNA using TAKARA RNA PCR kit (TAKARA BIO, Shiga, Japan). Real time PCR was conducted with the LineGene33 (BioFlux, Tokyo, Japan) using SYBR Green Realtime PCR Master Mix (TOYOBO Co, Osaka, Japan). As an internal control measurement, to normalize for input DNA, copy numbers of β-actin were determined in every sample tested. The ratio of the normalized mean value for drug-treated samples was calculated and is given in the graphs.

Measurement of adiponectin levels in culture medium

The secretion of adiponectin from 3T3-L1 cells were determined by measuring the adiponectin concentration of culture medium, using the Mouse Adiponectin/Acrp30 Immunoassay (R&D systems, Mimmeapolis, USA).

Oil Red O staining

The cellular lipid content was assessed by lipid staining with Oil Red O. Staining was quantified at 520 nm after