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CD8$^+$ T Cell Cross-Reactivity Profiles and HIV-1 Immune Escape towards an HLA-B35-Restricted Immunodominant Nef Epitope

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Abstract

Antigen cross-reactivity is an inbuilt feature of the T cell compartment. However, little is known about the flexibility of T cell recognition in the context of genetically variable pathogens such as HIV-1. In this study, we used a combinatorial library containing 24 billion octamer peptides to characterize the cross-reactivity profiles of CD8$^+$ T cells specific for the immunodominant HIV-1 subtype B Nef epitope VY8 (VPLRPMTY) presented by HLA-B*35:01. In conjunction, we examined naturally occurring antigenic variations within the VY8 epitope. Sequence analysis of plasma viral RNA isolated from 336 HIV-1-infected individuals revealed variability at position (P) 3 and P8 of VY8; Phe at P8, but not Val at P3, was identified as an HLA-B*35:01-associated polymorphism. VY8-specific T cells generated from several different HIV-1-infected patients showed unique and clonotype-dependent cross-reactivity footprints. Nonetheless, all T cells recognized both the index Leu and mutant Val at P3 equally well. In contrast, competitive titration assays revealed that the Tyr to Phe substitution at P8 reduced T cell recognition by 50–130 fold despite intact peptide binding to HLA-B*35:01. These findings explain the preferential selection of Phe at the C-terminus of VY8 in *HLA-B*35:01*$^+$ individuals and demonstrate that HIV-1 can exploit the limitations of T cell recognition *in vivo*.

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Introduction

Hypervariable viruses such as HIV-1 can escape from human leukocyte antigen class I (HLA-I)-restricted CD8$^+$ T cell responses by acquiring viral genomic mutations within or near immunogenic epitopes. Such immune escape pathways can be extremely reproducible and broadly predictable based on host HLA-I alleles at a population level [1,2]. Somewhat paradoxically, however, antigen cross-reactivity is an inbuilt feature of the T cell compartment [3,4]. Indeed, a single autoimmune T cell receptor (TCR) has recently been shown to recognize more than a million different peptides within a broad cross-reactivity profile encompassing unrelated amino acid substitutions [5]. Furthermore, several lines of evidence suggest that certain CD8$^+$ T cell subsets with the capacity to cross-recognize naturally occurring viral variants are advantageous for viral control *in vivo* [6–11]. However, the true extent of HIV-1-specific T cell cross-reactivity remains elusive. In the present study, we characterized the cross-reactivity footprints of HIV-1-specific CD8$^+$ T cells using combinatorial peptide library (CPL) scanning to cover all possible amino acid variations at each position of an octamer epitope. Additionally, we analyzed antigenic variation within the targeted epitope region of HIV-1 subtype B. Our investigations focused on CD8$^+$ T cell responses specific for the immunodominant HIV-1 Nef epitope VY8 (VPLRPMTY) presented by HLA-B*35:01 [12,13].

Materials and Methods

Ethics Statement

All study participants provided informed, written consent at the AIDS Clinical Center, National Center for Global Health and Medicine, Japan. The study was approved by the Institutional Review Board of Kumamoto University and National Center for Global Health and Medicine.

Sequence Analysis of Autologous HIV-1

Treatment-naïve individuals (n = 336) with chronic HIV-1 infection (>90% subtype B) attending the AIDS Clinical Center (International Medical Center of Japan) were enrolled for autologous HIV-1 sequence analysis. The median [IQR] plasma viral load was 95,000 [31,000–350,000] copies/ml; the median

Table 1. TCR β composition of CD8+ T cell lines.

Patient	β chain			
	V gene	J gene	CDR3 sequence	Frequency
Pt-100	BV2*01	BJ2-7*01	CASSGEGNYEQYF	1/31
			CASTTDRVYEQYF	1/31
	BV3-1*01	BJ2-5*01	CASSTSSVTETQYF	2/31
		BJ2-7*01	CASSQDIAGVHEQYF	1/31
	BV4-1*01	BJ2-1*01	CASSQTSGSYNEQFF	1/31
	BV6-1*01	BJ1-5*01	CASSEASGIYEQYF	1/31
		BJ2-7*01	CASSEASGIYEQYF	1/31
	BV10-1*01	BJ2-1*01	CASSAAGVEYNEQFF	1/31
	BV11-2*01	BJ1-1*01	CASSFDIVNTEAFF	1/31
		BJ2-1*01	CASSPDLVDNEQFF	4/31
		BJ2-5*01	CASSGAWTGGGETQYF	2/31
		BJ2-7*01	CASSLDLVSYEQYF	1/31
			CASSLGIGRAYEQYF	1/31
	BV12-3*01	BJ1-4*01	CASSLRFATNEKLFF	1/31
	BV27*01	BJ2-5*01	CASSFDTNQETQYF	1/31
		BJ2-7*01	CASSLDTNGYEQYF	1/31
			CASSFQLAGVHGQYF	1/31
			CASSPRLDDEQYF	2/31
			CASSLDTSGYEQYF	2/31
			CASSSDREDSHEQYF	2/31
	BV28*01	BJ2-2*01	CASSSTDRAIPNTGELFF	1/31
		BJ2-3*01	CASSLPGLDSTDTQYF	1/31
		BJ2-7*01	CASSEGQGRYEQYF	1/31
Pt-168	BV2*01	BJ2-7*01	CASSESLAGGPYEQYF	7/31
	BV3-1*01	BJ2-3*01	CASSQEGADTQYF	2/31
	BV3-1*02	BJ2-3*01	CASSQEGAGTQYF	1/31
	BV6-2*01	BJ1-1*01	CASSGGRTDENTEAFF	1/31
		BJ2-1*01	CASSYEREDSGNEQFF	1/31
	BV11-2*01	BJ2-7*01	CASSLDVAGSYEQYF	1/31
			CASSLDIVSYEQYF	1/31
	BV11-3*03	BJ2-3*01	CASSLVLGTGTDTQYF	1/31
	BV12-3*01	BJ2-3*01	CASSWDSISTDTQYF	1/31
		BJ2-7*01	CASSSDGYEQYF	3/31
	BV12-5*01	BJ2-2*01	CASGLAMVVSGELFF	1/31
	BV15*02	BJ2-1*01	CATSRDLVEDEQFF	2/31
	BV20-1*05	BJ2-2*01	CSARDPRTDRGNTGELFF	1/31
	BV24-1*01	BJ2-3*01	CATSVRDDLTGNGPDTQYF	2/31
	BV27*01	BJ2-3*01	CASSLDLRPDTQYF	1/31
	BV28*01	BJ2-5*01	CASSLLGEETRETQYF	4/31
	BV30*01	BJ2-5*01	CAWHTVRVQETQYF	1/31

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[IQR] CD4+ T cell count was 242 [64.5–367.5] cells/mm^3. We determined autologous *nef* sequences from plasma viral RNA using a previously reported direct sequencing method [13].

Table 2. TCR β composition of CD8+ T cell clones.

Patient	Clone	β chain		
		V gene	J gene	CDR3 sequence
Pt-19	19-136	BV7-2*03	BJ2-1*01	CASSPTPQGDYEQFF
	19-139	BV11-2*01	BJ1-1*01	CASSLDLVSTEAFF
Pt-33	33-S1	BV4-2*01	BJ2-3*01	CASSQAADAAITDADTQYF
Pt-100	100-K51	BV27*01	BJ2-5*01	CASSFDTNQETQYF
	100-K105	BV11-2*01	BJ1-1*01	CASSFDIVNTEAFF
	100-K810	BV27*01	BJ2-7*01	CASSFQLAGVHGQYF

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Generation and Maintenance of CD8+ T cell Lines and Clones

The CD8+ T cell clones (19–136, 19–139 and 33-S1) were established previously [13]. Additional CD8+ T cell lines and clones were generated by VY8 peptide stimulation of peripheral blood mononuclear cells (PBMCs) isolated from *HLA-B*35:01*+ individuals with chronic HIV-1 infection (Pt-100 and Pt-168) with 10 nM of VY8 (VPLRPMTY) peptide. The Institutional Review Board of the National Center for Global Health and Medicine approved both taking samples and generating cell lines, and patients provided the written informed consent. All CD8+ T cell lines and clones were maintained in RPMI 1640 supplemented with 10% fetal calf serum, 10 IU recombinant human interleukin (IL)-2, antibiotics and L-glutamine.

Analysis of TCR-encoding Genes

TCR-encoding genes of CD8+ T cell lines and clones were obtained by using a SMART PCR cDNA synthesis kit (Clontech) and analyzed with reference to the ImMunoGeneTics database (http://imgt.cines.fr) as described previously [14].

T cell Sensitivity Assay

Secretion of cytokines and chemokines by virus-specific CD8+ T cells in response to specific antigen provides a useful tool for quantitative assessment of antigen recognition [15,16]. MIP-1β was used as a functional readout in this study since it is one of the most sensitive means to assess functional avidity of human CD8+ T cells as previously described [15–17]. Briefly, 3×10^4 T cells were mixed with 6×10^4 HLA-B*35:01-expressing C1R cells (C1R-B3501), either unpulsed or pulsed with cognate peptide across a range of concentrations. After overnight incubation at 37°C, the supernatant was harvested and assayed for MIP-1β content by ELISA as described previously [5,17]. The amount of MIP-1β released in the absence of the peptide was subtracted as background. It should be noted that the VY8 peptide titration experiments of T cell clones 136 and 139 exhibited comparable results when IFN-γ [13] and MIP-1β were used as readouts (data not shown).

Figure 1. Amino acid residues preferentially recognized by VY8-specific CD8$^+$ T cells. Graphical representation showing relative preference for amino acid residues recognized by VY8-specific T cell lines and clones based on the CPL scan data shown in Figure S1. Responses >20% were included. A web-based application, WebLogo 3 (http://weblogo.threeplusone.com/), was used to generate the graphic. Colours represent physicochemical properties: polar (G, S, T, Y and C), green; neutral (Q and N), purple; basic (K, R and H), blue; acidic (D and E), red; hydrophobic (A, V, L, I, P, W, F and M), black. The index residues at each position are outlined in yellow. Residue size is proportional to T cell recognition preference.
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Octamer Combinatorial Peptide Library (CPL) Scan

The octamer CPL contained a total of 2.4×10^{10} different peptides (PepScan) divided into 160 sub-mixtures in positional scanning format as described previously [4,18]. Target C1R-B3501 cells (6×10^4 cells/well) were pre-incubated in the absence or presence of CPL sub-mixtures (100 µg/ml). Effector T cells (3 x 10^4 cells/well) were then added and incubated overnight at 37°C. Supernatant was collected and analyzed for MIP-1β content by ELISA as described previously [5,17]. Background-subtracted results were expressed as % response, normalized with respect to the VY8 index residue. A response >20% was considered positive.

Results and Discussion

Clonotypic Characterization of VY8-specific T cells

CD8$^+$ T cell lines were established from two *HLA-B**35:01*$^+$ individuals with chronic HIV-1 infection (Pt-100 and Pt-168). Analysis of TCR β usage by these T cell lines revealed multiple clonotypes, with 23 and 17 distinct TCR β sequences for Pt-100 and Pt-168, respectively (Table 1). This observation is consistent with previous studies showing the oligoclonal nature of immunodominant HIV-1-specific CD8$^+$ T cell populations [19,20]. The CD8$^+$ T cell clones K51, K105 and K810 were generated from patient Pt-100 by limiting dilution of VY8-specific T cell lines. Monoclonality was confirmed by TCR β analysis and all three sequences were encompassed within the TCR repertoire of the parental T cell lines (Table 2). Additional CD8$^+$ T cell clones (136, 139, and S1) previously established from two separate *HLA-B**35:01*$^+$ HIV-1-infected individuals [12,13] showed distinct TCR β chain usage (Table 2) and were also used for cross-reactivity studies.

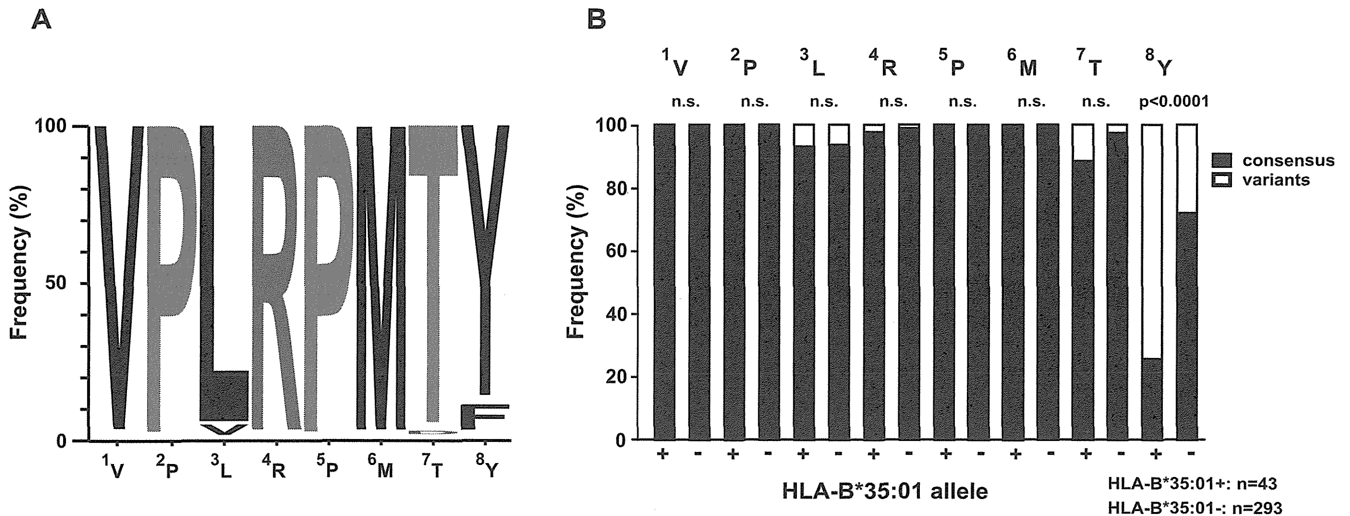

Figure 2. Naturally arising antigenic variations in the VY8 epitope. (**A**) Graphical representation showing the frequency of amino acid residues within the VY8 epitope in subtype B Nef sequences retrieved from the Los Alamos database (n = 1191). WebLogo 3 was used to generate the graphic. (**B**) The frequency of consensus (subtype B) and variant amino acid residues at each position of the VY8 epitope is shown for autologous plasma viral sequences derived from a total of 336 HIV-1-infected individuals, segregated according to *HLA-B*35:01* status. Statistical analysis was performed using Fisher's exact test. *n.s.*, not significant.
doi:10.1371/journal.pone.0066152.g002

Cross-reactivity Analysis of VY8-specific T cells

The cross-reactivity profiles of VY8-specific T cell lines and clones were analyzed using a CPL containing a total of 2.4×10^{10} different octamer peptides, which allowed qualitative mapping of preferred T cell recognition residues at each position along the peptide backbone [4,18]. Different VY8-specific T cell lines and clones preferentially recognized different amino acid residues across the octamer peptide backbone (Figure S1). We employed a graphical representation of these preferential recognition residues by the VY8-specific T cells (Figure 1). Despite these unique cross-reactivity patterns, all T cells tested recognized the index VY8 residues efficiently (Figure 1). This finding contrasts with previous observations using tumor-specific and autoreactive T cell clones [5,21–23], which typically prefer non-index amino acid residues. Across all clones, more stringent recognition was observed at position 2 (P2) and P8 (Figure 1). This most likely reflects the anchor role of these positions in peptide binding to HLA-B*35:01 [12,24]. The VY8-specific T cell clones, K51, K105 and K810, showed inherently unique cross-reactivity footprints but less flexible cross-recognition compared to the parental T cell line (Figure 1), suggesting increased coverage of viral antigenic variation through polyclonal TCR cross-reactivity.

Naturally Occurring Antigenic variations within the VY8 Epitope

To investigate the correlation between T cell cross-reactivity and naturally occurring antigenic variation, we analyzed sequence polymorphisms within the VY8 epitope. Despite the remarkable variability of HIV-1 Nef, VY8 is highly conserved, most likely due to its location partially within a Src homology 3 binding motif that

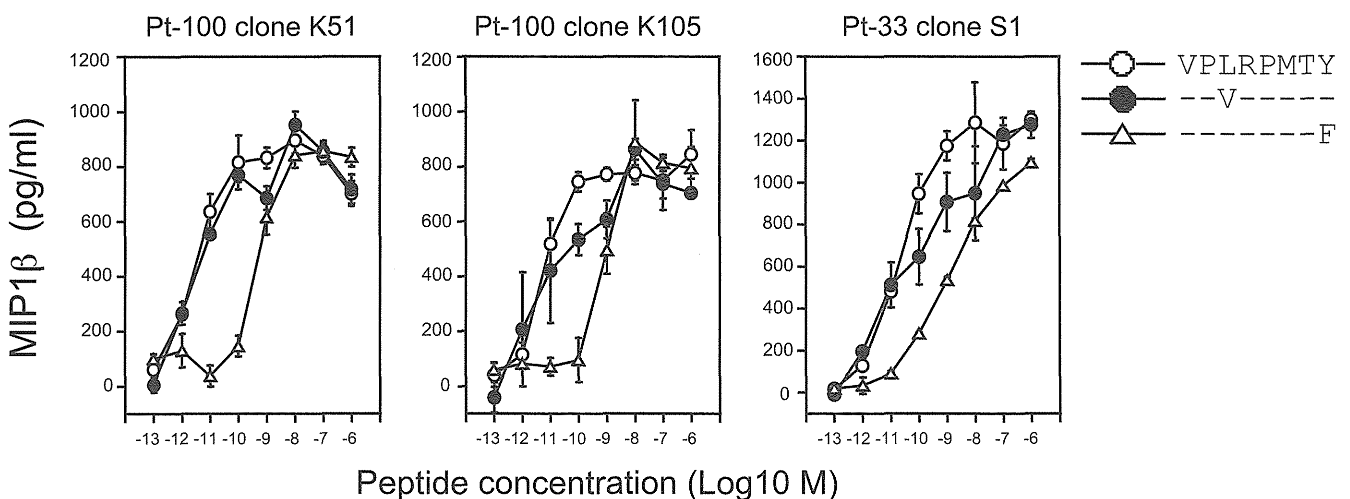

Figure 3. VY8-specific CD8+ T cell sensitivity towards peptide variants. The sensitivity of T cell clones towards the VY8, VY8-3V and VY8-8F peptides was quantified by measuring the amount of MIP-1β secreted in response to antigen stimulation. Data are representative of duplicate assays and standard deviation from the mean of two replicates is shown.
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Table 3. Sensitivity of VY8-specific $CD8^+$ T cells.

$CD8^+$ T cells		EC_{50} (M)		
		VY8	VY8-3V	VY8-8F
lines	Pt-100	5.9×10^{-12} (x 1)	nd	3.9×10^{-10} (x 66)
	Pt-168	4.0×10^{-12} (x 1)	nd	4.3×10^{-10} (x 105)
clones	33-S1	2.3×10^{-11} (x 1)	3.9×10^{-12} (x 0.17)	1.2×10^{-9} (x 52)
	100-K51	3.1×10^{-12} (x 1)	5.8×10^{-12} (x 1.8)	4.2×10^{-10} (x 135)
	100-K105	5.1×10^{-12} (x 1)	3.9×10^{-12} (x 0.76)	6.7×10^{-10} (x 131)

EC_{50}, determined by duplicate assays; nd, not done; in parenthesis, fold changes in sensitivity relative to index.
doi:10.1371/journal.pone.0066152.t003

is critical for several Nef functions [25], including HLA-I down-regulation [13,26]. Nevertheless, in the Los Alamos HIV Sequence database (http://www.hiv.lanl.gov/content/index), some variability within HIV-1 subtype B has been reported at P3 Leu and P8 Tyr of the VY8 epitope, with 2.4% and 8.2% of viral clones showing polymorphisms in these positions, respectively (Figure 2A). Given that approximately 40% of Nef sequence polymorphisms are associated with host HLA-I alleles [1], we examined these particular variants for HLA-I association. Our previous smaller study of 69 HIV-1-infected patients indicated that Phe at P8 might be associated with the *HLA-B*35:01* allele [13]. To confirm this association and examine polymorphisms at P3, we recruited a larger cohort comprising 336 treatment-naïve individuals with chronic HIV-1 infection and determined autologous *nef* sequences from plasma viral RNA. Although we found some variability at P3 (3%), there were no statistically significant amino acid differences at P1–P7 between individuals with or without *HLA-B*35:01* (Figure 2B). In fact, CPL scanning showed that, at P3, hydrophobic residues including both the index Leu and mutant Val were preferentially recognized by all VY8-specific T cells tested (Figure 1). Such flexible TCR recognition at P3 helps to explain why the Val mutant is not selected in *HLA-B*35:01*$^+$ individuals. Conversely, we found a statistically significant difference in the frequency of polymorphisms at P8 between individuals with or without *HLA-B*35:01* (Figure 2B); indeed, the vast majority (74%) of *HLA-B*35:01*$^+$ donors harboured viral sequences with Phe at P8. However, CPL scanning showed that Phe was a favoured amino acid residue recognized by T cell lines and some clones, such as K105 (Figure 1 and Figure S1). In these instances, CPL data alone do not simply explain the emergence of this viral mutation in *HLA-B*35:01*$^+$ individuals.

VY8-specific T cell Sensitivity Towards Peptide Variants

To verify the effect of single mutations within the VY8 peptide on TCR sensitivity, we performed competitive titration assays across our panel of VY8-specific T cells (Figure 3). Consistent with the CPL scan data, all T cells tested recognized the VY8 and VY8-3V peptides comparably (<2 fold difference in EC_{50} values; Table 3). In contrast, the EC_{50} values for VY8-8F were >50 fold higher than index for all T cells tested (Table 3). These observations are consistent with previous reports showing that VY8-specific T cells could not recognize $CD4^+$ T cells or macrophages infected with HIV-1 carrying this Nef variant at P8 [13,26].

Although P8 is an anchor residue for VY8, our previous HLA-I stabilization studies showed comparable binding activity between HLA-B*35:01 and either VY8 or VY8-8F [13]. The crystal structure of the VY8/HLA-B*35:01 complex shows that P8 Tyr lies deep inside the F pocket of the HLA-I molecule [24]. Substitution at this position with the aromatic residue Phe may not induce substantial structural changes. Consequently, impaired T cell recognition of P8 Phe may be mediated by indirect conformational changes imposed by the peptide upon TCR binding [17]. In the context of HLA-A*02:01, however, a Tyr to Phe substitution at the secondary anchor P3 of an antigenic peptide (SLFNTVATL) leads to unexpectedly large conformational changes in the peptide backbone [27]. Accordingly, further structural studies are needed to elucidate the precise mechanism through which anchor residue substitution leads to impaired T cell recognition of the VY8 epitope.

Previous studies have shown that the double substitution of Arg-71 to Thr and Tyr-81 to Phe (P8 at VY8) [13], or Pro-75 to Ala (P2 at VY8) as a single mutation, impair Nef-mediated down-regulation of HLA-I and thereby increase the susceptibility of HIV-1-infected cells to killing by $CD8^+$ T cells targeting other epitopes [26,28]. In contrast, the Tyr-81 to Phe (P8 at VY8) mutation alone exerts virtually no effect on Nef-mediated activities [13,26]. Collectively, these data suggest that the P8 Phe mutation does not compromise viral fitness.

Concluding Remarks

$CD8^+$ T cell responses against the immunominant HIV-1 subtype B-derived Nef epitope VY8 presented by HLA-B*35:01 are highly polyclonal, broadly cross-reactive and capable of tolerating natural viral variation with one notable exception. Specifically, the observed Phe substitution at P8, which is neutral in terms of Nef-mediated function [13,26], was found to reduce $CD8^+$ T cell recognition by >50 fold. The association of this mutation with *HLA-B*35:01*$^+$ strongly suggests that evasion of VY8-specific $CD8^+$ T cell activity confers a selection advantage *in vivo*. Thus, even $CD8^+$ T cell responses with extensive cross-reactivity profiles can succumb to immune escape at a single position.

Supporting Information

Figure S1 CPL scanning of VY8-specific $CD8^+$ T cells. The cross-reactivity profiles of T cell lines and clones specific for VY8 were tested by using 160 CPL sub-mixtures (100 µg/ml) comprising a total of 2.4×10^{10} different octamer peptides. In every peptide mixture, one position has a fixed amino acid residue and all other positions are degenerate, with the possibility of any one of 19 natural amino acids being incorporated in each individual position (cysteine is excluded). The amount of MIP-1β secreted in response to antigen was quantified by ELISA. Data are background-subtracted and the relative T cell response is shown as a ratio of MIP-1β production with respect to the index residue at each position. Responses >20% were considered positive and used to construct Figure 1. A representative set of duplicate assays is shown. Red bars depict residues corresponding to the VY8 index sequence.
(EPS)

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Author Contributions

Conceived and designed the experiments: CM JJM AKS TU. Performed the experiments: CM JJM ZH SCM TU. Analyzed the data: CM JJM ZH

SCM DAP AKS TU. Contributed reagents/materials/analysis tools: HG SO. Wrote the paper: CM JJM DAP AKS TU.

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Research Letters

AIDS 2013, **27**:839–848

Once-daily darunavir/ritonavir and abacavir/lamivudine versus tenofovir/emtricitabine for treatment-naïve patients with a baseline viral load of more than 100 000 copies/ml

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The efficacy and safety of fixed-dose abacavir/lamivudine against tenofovir/emtricitabine, both with once-daily darunavir/ritonavir, was examined in 80 treatment-naïve patients with a baseline HIV-1 viral load of more than 100 000 copies/ml. The time to virologic failure by 48 weeks was not different between the two groups. The percentage of patients with viral suppression was not significantly different with per protocol population. Tenofovir/emtricitabine showed better tolerability; more patients on abacavir/lamivudine changed regimen than those on tenofovir/emtricitabine. A randomized trial to elucidate the efficacy and safety of these two regimens is warranted.

Little information is available on the efficacy and safety of antiretroviral therapy (ART) of ritonavir-boosted darunavir (DRV/r) and fixed-dose abacavir/lamivudine (ABC/3TC) [1,2]. DRV/r is a protease inhibitor with proven efficacy and safety, and with high barrier to drug resistance [3,4]. ABC/3TC is an alternative choice of nucleoside reverse transcriptase inhibitors (NRTIs) in the American Department of Health and Human Services Guidelines [5]. Here, we conducted a single-center, observational pilot study to compare the efficacy and safety of DRV/r and ABC/3TC versus tenofovir/emtricitabine (TDF/FTC) in patients with a baseline HIV-1 viral load of more than 100 000 copies/ml. Patients with such a viral load were chosen because ACTG 5202 demonstrated that the time to virologic failure was significantly shorter with ABC/3TC than with TDF/FTC in patients with a viral load of more than 100 000 copies/ml on efavirenz or ritonavir-boosted atazanavir [6]. All patients were treatment-naïve who commenced once-daily DRV/r and either fixed-dose ABC/3TC or TDF/FTC from November 2009 to August 2011 at the AIDS Clinical Center, Tokyo. Baseline data (basic demographics, CD4 count, and viral load) were collected. Viral load was measured by Cobas TaqMan HIV-1 real-time PCR version 1.0 assay (Roche Diagnostics, NJ) to the end of November 2011, and later by Cobas TaqMan version 2.0 assay. It was the decision of the attending physician to start ART with either TDF/FTC or ABC/3TC, because the Japanese guidelines consider both TDF/FTC and ABC/3TC as the preferred NRTIs [7].

The efficacy outcomes were the time from commencing ART to virologic failure (defined as a viral load > 1000 copies/ml at or after 16 weeks and before 24 weeks, or >200 copies/ml at or after 24 weeks) [6], and the proportion of patients with a viral load < 50 copies/ml at 48 weeks regardless of previous virologic failure. The tolerability outcome was the time to any regimen modification. Intent-to-treat (ITT) population, comprising all patients, was used for all efficacy and tolerability analyses, whereas per protocol population was used in the efficacy analysis of the suppressed viral load. Censored cases represented those who dropped out, referred to other facilities, or reached 48 weeks. Time-to-event distributions were estimated using the Kaplan–Meier method. Univariate and multivariate Cox hazards models estimated the impact of ABC/3TC use over TDF/FTC on the incidence of virologic failure.

The study included 80 patients [ABC/3TC: 21, TDF/FTC: 59, median age: 37.9 years, men: 74 (92.5%), East Asian origin: 72 (90%)], of whom 66 (82.5%) were infected with HIV-1 through homosexual contact. Patients on ABC/3TC had a lower baseline CD4 count (46/μl versus 100, $P = 0.031$), higher viral load (5.75 $\log_{10}$ copies/ml versus 5.58, $P = 0.044$), and were more likely to have a history of AIDS (71.4% versus 37.3, $P = 0.010$), than patients with TDF/FTC. All subjects were HLA-B*5701-negative, and all underwent HIV-1 drug-resistance tests before commencement of ART and none had resistant mutations.

The time to virologic failure with ABC/3TC [3 patients (14.3%)] was not significantly different from that with TDF/FTC [4 (6.8%)] by 48 weeks (Fig. 1a), by univariate and multivariate analyses adjusted by CD4 count and viral load (HR, 2.651; 95% CI, 0.592–11.88; $P = 0.203$, adjusted HR, 1.589; 95% CI, 0.341–7.401; $P = 0.555$). At week 48, ITT analysis showed more patients with TDF/FTC had a viral load of less than 50 copies/ml (ABC/3TC: 38.1%, TDF/FTC: 64.4%, $P = 0.043$) (Fig. 1c), whereas with per protocol analysis, no difference was noted (ABC/3TC: 57.1%, TDF/FTC: 73.1%, $P = 0.328$) (Fig. 1d).

Among the seven patients with virologic failure, three (ABC/3TC: 1, TDF/FTC: 2) achieved sustained viral

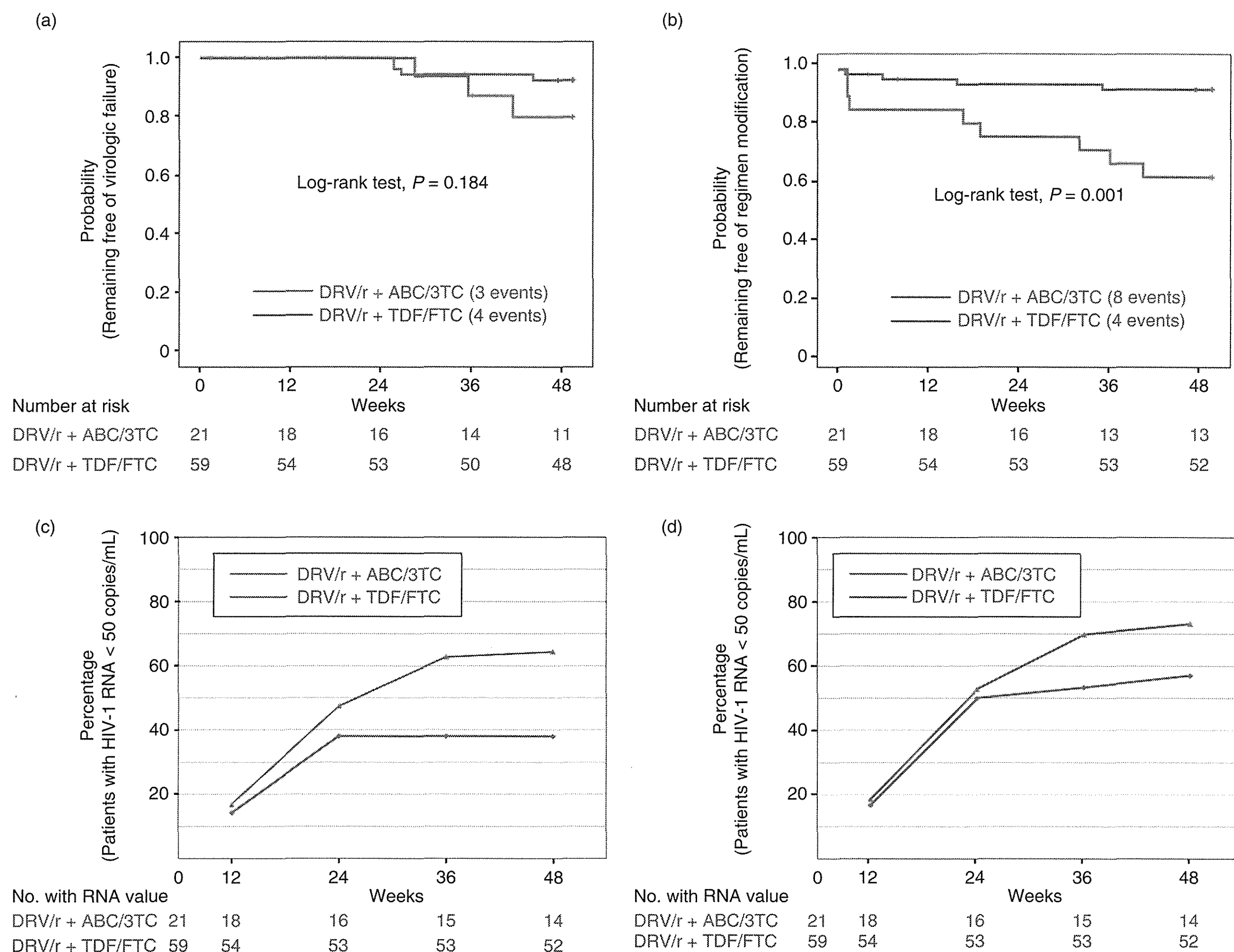

Fig. 1. Efficacy and tolerability results over 48 weeks. (a) Time to protocol-defined virologic failure. (b) Time to tolerability endpoint, defined as first change in treatment regimen. Percentage of patients with HIV-1 RNA less than 50 copies/ml at weeks 12, 24, 36, and 48, regardless of previous virologic failure, with (c) intention-to-treat population, and with (d) per protocol population.

load suppression after week 60 of the initial regimen. The other four underwent drug-resistance tests. One on ABC/3TC was switched to TDF/FTC at week 41; however, viral suppression was not achieved until raltegravir was added at week 74. The other with ABC/3TC was switched to TDF/FTC at week 49 and achieved viral suppression despite the emergence of protease mutation M46I. Another patient on TDF/FTC had persistent viremia (100–200 copies/ml) without mutation. Another patient on TDF/FTC showed the emergence of reverse transcriptase mutation V75L and viremia persisted with 200–500 copies/ml. Reverse transcriptase mutation M184I/T/V did not emerge in any patients.

More patients on ABC/3TC changed or discontinued the initial regimen during the research period [ABC/3TC: 8 (38.1%), TDF/FTC: 4 (6.8%), $P = 0.001$] (Fig. 1b). Six [ABC/3TC: 4 (19%), TDF/FTC: 2 (3.4%)] changed ART due to adverse events or virologic failure [ABC/3TC: virologic failure ($n = 1$), limb paresthesia ($n = 1$), and nausea ($n = 2$); TDF/FTC: tenofovir nephrotoxicity ($n = 2$)]. None developed ABC-associated hypersensitivity.

This is the first comparison report of the efficacy and safety of ABC/3TC against TDF/FTC with DRV/r in treatment-naïve patients with a viral load of more than 100 000 copies/ml. The time to virologic failure by 48 weeks was not different between the two groups. Although a higher percentage of patients on TDF/FTC showed viral suppression than those on ABC/3TC at week 48 with ITT population, the difference was not significant with per protocol population. TDF/FTC showed better tolerability, as more patients on ABC/3TC changed regimen than those on TDF/FTC.

These results need to be interpreted with caution, because the baseline characteristics of patients of the two groups were not well matched due to the nature of the observational study, and this study did not have sufficient power due to the small number of enrolled patients.

Because our patients had small stature with median body weight of 58.1 kg, a risk factor for TDF nephrotoxicity, it is sometimes our practice to avoid TDF in patients with multiple risks, such as advanced HIV-1 infection, to prevent possible acute kidney injury [8–10]. This is presumably the reason for prescribing ABC/3TC to patients with worse disease condition in this study. This allocation bias might have worked as a disadvantage for the efficacy and tolerability results of ABC/3TC.

The usefulness of ABC/3TC has recently received higher recognition than it did in the past; the FDA meta-analysis did not confirm the association between ABC use and myocardial infarction [11], and it became clear that TDF use is associated with decreased bone mineral density and renal dysfunction, both of which might develop into serious complications with long-term TDF use [12–17]. Thus, once-daily DRV/r, a protease inhibitor with high barrier to drug resistance, and ABC/3TC could be good alternative, especially in patients, who cannot tolerate TDF. A randomized trial to elucidate the efficacy and safety of ABC/3TC and TDF/FTC with once-daily DRV/r is warranted.

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Conflicts of interest

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Poor outcome of HIV-infected patients with plasmablastic lymphoma: results from the German AIDS-related lymphoma cohort study

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Out of 302 AIDS-related lymphoma (ARL) patients enrolled in the German ARL cohort study, 18 patients had plasmablastic lymphoma (PBL). Twelve out of 18 patients (67%) have died with a median survival of 4 months (range 0–11 months). In univariate analysis, an intermediate or high international prognostic index score was associated with a significantly lower overall survival and progression-free survival. The predominant cause of death was progressive lymphoma (67%). Our data indicate that the outcome of AIDS-related PBL is still very poor.

Since the introduction of combination antiretroviral therapy (cART), the incidence of AIDS-related lymphomas (ARLs) has remarkably declined while the prognosis has considerably improved [1,2]. However, ARLs still remain a serious cause of mortality and morbidity in HIV-infected patients [3]. Plasmablastic lymphomas (PBLs), which are characterized by the absence of B-cell markers (CD20) and the presence of plasma cell markers, comprise a rare entity within ARL [4–8]. The aim of the present study was to describe the clinical characteristics and to analyze the outcome of HIV-infected patients with PBL enrolled in the prospective German ARL-cohort study.

The German ARL-cohort study is a prospective observational multicenter evaluation. HIV-1-infected patients with ARL diagnosed in 30 participating German centers after 1 January 2005, were included in the study. The present analysis consists of 18 patients with the histopathological diagnosis of PBL out of 302 ARL patients enrolled until June 2011. Fifteen out of 18 cases with diagnosis of PBL were confirmed by a review pathologist of one of the German lymphoma reference centers. Overall survival (OS) and progression-free survival (PFS) were calculated from the date of ARL diagnosis until death or until the last follow-up and until lymphoma progression or death as a result of any cause. Kaplan–Meier survivor function was used to evaluate OS and PFS. Prior AIDS-defining illness, CD4 T-cell count at ARL diagnosis, cART before ARL diagnosis, suppressed HIV-RNA, age more than 60, enhanced lactate dehydrogenase (LDH), Eastern Cooperative Oncology Group (ECOG) [9] score >2, stage III/IV disease, extranodal involvement, and the International Prognostic Index (IPI) [10] were considered as potential predictors (definitions of ECOG, IPI, and Ann Arbor score [11] are listed in Table 1). Approval was granted by the ethic committee of the University of Cologne, Germany and of each participating site. Written informed consent was obtained.

All patients were men with a median age of 44 years. Median CD4 T-cell count at ARL diagnosis was 85/μl (range 0–1100/μl). Only five patients had an undetectable HIV-RNA at the time of PBL diagnosis. The baseline characteristics are depicted in Table 1.

With regard to histopathological findings, all PBLs were CD20-negative and at least one plasma cell marker (VS38c, CD38, MUM1, CD138) has been expressed in 82% of cases. Data on KI-67 and Epstein–Barr virus (EBV) are available for 94 and 78% of cases, respectively. A very high proliferation index (KI-67 ≥80%) was found in 13 out of 17 patients (76%) and EBV positivity was observed in 12 out of 14 cases (86%).

Protocols based on CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone) were the initial regimen (CHOP-21: $n=6$, CHOP-14: $n=3$, CHOEP: $n=1$) in 10 patients, whereas seven patients were treated according to the high-dose methotrexate-based B-ALL protocol adapted from B-ALL/NHL2002 (ClinicalTrials.gov identifiers NCT00199082/NCT00388193) of the German Multicenter Study Group for the Treatment of Adult Acute Lymphoblastic Leukemia (GMALL). Twelve patients (67%) received at least four cycles of chemotherapy according to the CHOP protocol or B-ALL protocol.

By 30 June, 2011, 12 out of 18 patients (67%) have died after a median survival time of 4 months (range 0–11 months; Table 1). None of these patients achieved a complete remission. Six patients were still alive in their first complete remission with a median follow-up of 32 months (range 21–76 months). The median survival of the entire cohort of patients was 5 months (range 0–76 months). By univariate analysis, an increased LDH, an ECOG performance >2, an age >60 years at lymphoma diagnosis, and an intermediate or high IPI

INTERNAL MEDICINE

□ ORIGINAL ARTICLE □

Epstein-Barr Viral Load in Cerebrospinal Fluid as a Diagnostic Marker of Central Nervous System Involvement of AIDS-related Lymphoma

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Abstract

Objective AIDS-related lymphoma (ARL) often involves the central nervous system (CNS). Although the diagnostic value of Epstein-Barr virus (EBV)-DNA in cerebrospinal fluid (CSF) in detecting HIV-positive primary CNS lymphoma (PCNSL) has been established, its usefulness for identifying CNS involvement of systemic ARL remains elusive. In this study, we evaluated the utility of the EBV-DNA load in CSF in identifying CNS involvement in patients with systemic ARL.

Methods We retrospectively reviewed the clinical and pathological data of consecutive ARL patients managed at our clinic between January 1998 and June 2012. Sixty-two patients with ARL, including eight PCNSL patients and 52 systemic ARL patients, and 63 controls underwent CSF EBV-DNA load evaluations before receiving chemotherapy. ARL-related CNS involvement was defined as any lesion diagnosed histologically or radiologically as a lymphoma in the brain, meninges, spine, cranial nerves or oculus.

Results A cut off value of 200 copies/mL predicted the presence of CNS lesions with a sensitivity of 70% and a specificity of 85% in both the PCNSL and systemic ARL patients, while a sensitivity of 75% and a specificity of 93% were obtained for systemic ARL. A cut off value of 2,000 (3.30 log) copies/mL provided the best specificity (100%), with a sensitivity of 50%.

Conclusion Our results support the clinical utility of evaluating the quantitative EBV-DNA load in the CSF for the diagnosis of CNS involvement of systemic ARL as well as PCNSL.

Key words: AIDS-related lymphoma, Epstein-Barr virus

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Introduction

Although the incidence of AIDS-related lymphoma (ARL) has decreased following the advent of highly active antiretroviral therapy (HAART), the morbidity and mortality associated with this complication remain significant due to the aggressive clinical course and high frequency of extra nodal localization especially in the central nervous system (CNS) (1-3). Since the majority of patients with ARLs are diagnosed at the advanced stage of HIV infection, making the differential diagnosis of CNS lesions from other opportunistic diseases is crucial for the management of ARL.

Epstein-Barr virus (EBV) can cause various lymphoproliferative disorders in immunocompromised patients and the detection of EBV-DNA in the cerebrospinal fluid (CSF) is a well-established diagnostic tool for identifying primary CNS lymphoma (PCNSL) in HIV-infected individuals (3-10). However, the diagnostic value of detecting EBV-DNA in CNS involvement of systemic ARL remains to be elucidated. In this study, we retrospectively evaluated the value of EBV-DNA in the diagnosis of CNS lesions of ARL, both PCNSL and systemic ARL.

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Table. Characteristics of the Participating Patients

	PCNSL (n=8)	Systemic ARL		non-ARL control subjects (n=63)	p value
		CNS involvement (+) (n=12)	CNS involvement (-) (n=42)		
Male sex, n	8	10	41	60	0.981
Age, median years (range)	38 (28-53)	52 (27-67)	37 (25-63)	38 (22-70)	0.160
Histology					
DLBCL	3	6	16	-	
Burkitt	0	4	16	-	
Others	2	1	10	-	
Not specified	3	1	0	-	
EBER-positive, % (n/total n)	40 (2/5)	40 (4/10)	58.3 (21/36)	-	0.999
CD4 count, median cells/mm^3 (range)	18 (2-79)	83 (3-652)	117 (3-824)	57 (1-450)	0.006
Plasma HIV viral load, median log copies/mL (range)	5.8 (4.5-6.0)	4.7 (1.6-7.1)	4.7 (1.6-7.5)	4.6 (1.7-6.3)	0.081
Plasma EBV-DNA-positive, % (n/total n)	66.7 (4/6)	63.6 (7/11)	58.3 (21/36)	NA	0.999
CSF EBV-DNA-positive, % (n/total n)	62.5 (5/8)	75.0 (9/12)	7.1 (3/42)	20.6 (13/63)	0.035

PCNSL: primary CNS lymphoma, ARL: AIDS-related lymphoma, DLBCL: diffuse large B-cell lymphoma, EBER: EBV-encoded small RNAs, NA: not assessed. CSF: cerebrospinal fluid. The Kruskal-Wallis test was used for comparisons of continuous variables and the chi-square test was used for comparisons of the categorical data.

Materials and Methods

We reviewed the clinical and pathological data of consecutive cases of ARL managed at the AIDS Clinical Center, National Center for Global Health and Medicine (NCGM), Tokyo between January 1998 and June 2012. CNS involvement of systemic ARL was defined as any lesion histopathologically or radiologically diagnosed as a lymphoma in the brain, meninges, spine, cranial nerves or oculus on either initial diagnosis or recurrence. HIV-infected patients with other opportunistic infections and meningeal or parenchymal brain lesions during the same period were enrolled in the control group for the analysis. Patients who did not have available CSF samples were excluded.

Real-time polymerase chain reaction (RT-PCR) was used to quantify EBV-DNA in CSF samples obtained before chemotherapy and stored at -80℃, using a method previously described (11). Briefly, DNA was extracted using a QIA Symphony Virus/Bacteria Mini kit (Qiagen, Valencia, CA), and the *BNRF1* gene was amplified with the following primers: forward [5'-CCAGTGCTGTGATCGAGCATCT] and reverse [5'-CTGTCGACAAACTGCTGCATTC] and TaqMan probe [5'-(FAM)-TCTGCTGTGTTTCTGTCTCACCTACCG G-(TAMRA)-3']. The cutoff level for detection was 200 copies/mL.

In patients with available results of *in situ* hybridization (ISH) assay of EBV-encoded small RNAs (EBERs), which were performed on paraffin tissue sections using a cocktail of fluorescein-isothiocyanate-labeled oligonucleotides complementary to the two EBERs (types 1 and 2), as previously described (12, 13), we assessed the correlation between the results of EBER and the CNS localization of lymphoma.

Before the analysis, the levels of EBV-DNA were log-transformed and samples with undetectable EBV-DNA were considered to contain 0.0 copies/mL. For continuous variables, the Mann-Whitney U-test was used to compare two groups, while the Kruskal-Wallis test was applied to compare three or more groups. Categorical data were examined using the chi-square test. Differences were considered to be significant at $p<0.05$. The statistical analyses were performed using the SPSS-II software package for Windows, version 17.0J (SPSS Japan Inc, Tokyo, Japan).

Results

During the study period, 76 patients were diagnosed with ARL, including eight patients with PCNSL and 68 patients with systemic ARL. One patient developed ARL twice (diffuse large B-cell lymphoma and plasmablastic lymphoma) within a several year interval and was considered to represent two systemic ARL cases. The frequency of CNS involvement in the systemic ARL patients was 22.1% (15/68). Of the 76 patients with ARL, 62 had available CSF samples and were assigned to the analysis [PCNSL n=8, systemic ARL with CNS involvement (ARL-CNS(+), n=12) and systemic ARL without CNS involvement (ARL-CNS(-), n=42)] (Table). The 63 control subjects with definitive diagnoses of other CNS opportunistic infections during the study period consisted of 18 patients with cryptococcal meningitis, 16 patients with toxoplasmosis, 12 with progressive multifocal leukoencephalopathy (PML), five patients with cytomegalovirus (CMV) encephalitis, three patients with tuberculous meningitis, three patients with neurosyphilis, three patients with Varicella-zoster virus meningitis, two patients with HIV encephalitis, one patient with aseptic meningitis due to acute retroviral syndrome and one patient with CNS candidiasis. Three subjects in the control group had multiple opportunistic infections. There were no significant differences in sex, age or HIV viral load between the two groups. The median CD4 count in the PCNSL group was significantly lower than that observed in the group with systemic ARL with CNS involvement; however, the CD4 counts of the other groups were comparable.

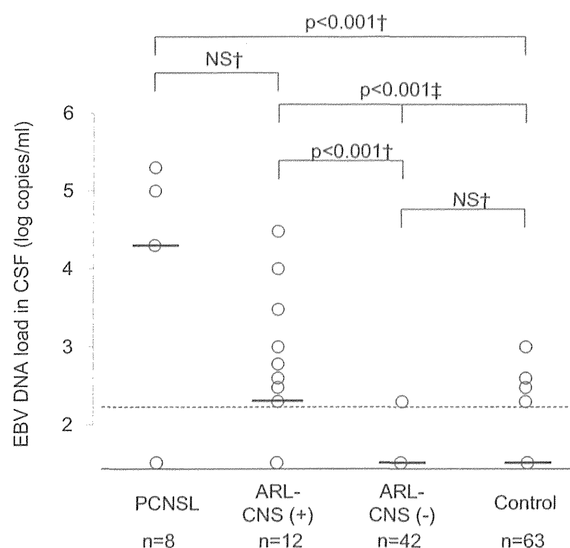

Figure 1. The EBV-DNA loads in the cerebrospinal fluid (CSF) of the patients with AIDS-related lymphoma and the control subjects. PCNSL: primary CNS lymphoma, ARL-CNS (+): systemic AIDS-related lymphoma with CNS involvement, ARL-CNS (-): systemic AIDS-related lymphoma without CNS involvement, NS: not significant. The Mann-Whitney U- test (†) and the Kruskal-Wallis test (‡) were used to compare to the EBV-DNA loads in the CSF. Individual values are plotted, and the horizontal bars represent the median values. The dotted horizontal line indicates the detection limit of the EBV-DNA load assay.

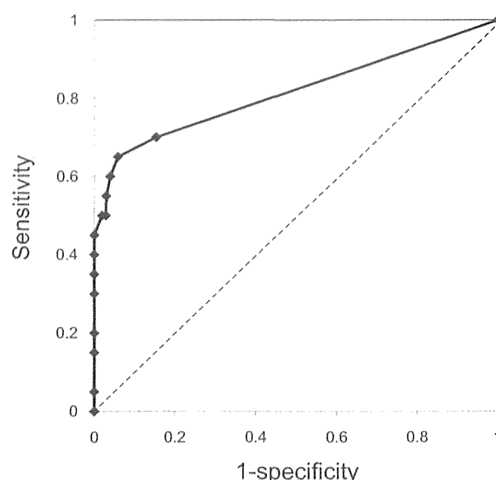

Figure 2. Receiver operating characteristic (ROC) curve for the cutoff values of the EBV-DNA load in the cerebrospinal fluid for the diagnosis of CNS involvement of systemic AIDS-related lymphoma. The dotted line is the reference line. The area under the ROC curve was 0.856 (95% confidence interval, 0.690-1.000). A cutoff value of 200 copies/mL had a sensitivity of 75% and a specificity of 93%.

The proportion of patients positive for EBV-DNA in the CSF (with a detection limit of 200 copies/mL) was 62.5% in the PCNSL, 75.0% in the ARL-CNS(+), 7.1% in the ARL-CNS(-) and 20.6% in the control group. The median (range) EBV-DNA loads in the CSF of the above groups were 4.30 (0-5.30), 2.53 (0-4.48), 0.00 (0-2.30) and 0.00 (0.00-3.00) log copies/mL, respectively (Fig. 1). Both the rate of EBV-DNA-positive cases (Table) and the median EBV-DNA load in the CSF (Fig. 1) were significantly higher in the PCNSL and ARL-CNS(+) groups compared with those observed in the ARL-CNS(-) and control groups; however, these values were not different between the PCNSL and ARL-CNS(+) groups or between the ARL-CNS(-) and control groups. Neither the detection of EBV-DNA in plasma nor histological evidence of EBER in tissue were found to be correlated with the CNS localization of lymphoma (Table). Among nine EBER-negative ARL-CNS(+) cases, CSF EBV-DNA was positive in the five patients who were positive for plasma EBV-DNA, while the remaining four patients were negative for both CSF and plasma EBV-DNA. Six EBER-positive ARL-CNS(+) cases included four patients with positive CSF EBV-DNA and negative plasma EBV-DNA, and one patients with positive and one patients with negative EBV-DNA in both the CSF and plasma. The concordant rate of EBV-DNA detection in the CSF and plasma was 100% in the EBER-negative in ARL-CNS(+) cases and 33% in the EBER-positive in ARL-CNS(+) cases.

With regard to the diagnostic value of the quantitative EBV-DNA load in the CSF, a cut off value of 200 copies/mL provided a sensitivity of 70% and a specificity of 85% for the CNS localization of all ARLs, including the cases of PCNSL and systemic ARL and provided a higher sensitivity of 75% and a specificity of 93% in the systemic ARL cases. A cut off value of 300 copies/mL exhibited a similar sensitivity of 65% and a higher specificity of 94%; however the best specificity (100%) was noted using a cut off value of 2,000 copies/mL, with a sensitivity of 50%. The area under the receiver operating characteristic (ROC) curve in the diagnosis of CNS localization of ARL was 0.816 for all ARLs and 0.856 for systemic ARLs (Fig. 2). Among the EBER-positive ARLs, a cut off value of 200 copies/mL provid a sensitivity of 83.3% and a specificity of 90.4% in the diagnosis of CNS involvement and provide a sensitivity of 55.6% and a specificity of 100% in the EBER-negative ARL cases.

Discussion

The present study demonstrated the usefulness of measuring the EBV-DNA load in the CSF for diagnosing CNS lesions of ARL, regardless of the type of localization of lymphoma, and the presence of PCNSL or CNS involvement of systemic ARL. Although the diagnostic value of EBV-DNA for HIV-positive PCNSL is well-documented (3-10, 18), evidence showing its usefulness for identifying CNS lesions of systemic ARL is limited (3-10). Since the prevalence (21.7%) of CNS involvement in patients with systemic ARL

is considerably higher (3) than that of non-HIV lymphoma patients (2-7%) (14-16), our results might support the clinical utility of evaluating EBV-PCR with CSF in the management of patients with HIV-positive systemic ARL.

In our study, quantitative EBV-PCR in the CSF with a cut off value of 200 (2.30 log) copies/mL had a sensitivity of 70% and a specificity of 85% for the identification of lymphoma in CNS, while a cut off value of 300 copies/mL provided a similar sensitivity of 65% and a higher specificity of 94%. A previous study that assessed the diagnostic value of quantitative EBV-DNA assays in the CSF for identifying both systemic ARL and PCNSL (10) reported a sensitivity of 75% and specificity of 76% using a cut off value of 100 copies/mL, while the best specificity (100%) was obtained using a cut off value of 3.53 log (3,388) copies/mL. Although our study used a slightly higher detection limit and had a higher specificity and lower sensitivity, the results of the two studies are comparable. In addition, a similar sensitivity (75%) and a higher specificity (93%) were obtained using the cut off value of 200 copies/mL for identifying CNS involvement in systemic ARL than from among all ARLs. Overall, a cut off value of 100-300 seems to be beneficial for identifying CNS lesions of ARL.

In the present study, the prevalence of CSF EBV-DNA in the PCNSL group (62.5%) and the EBER expression (40%) were relatively lower than those reported previously for AIDS-related PCNSL patients (80-100%) (3-10, 18). One possible reason for the low prevalence was the undetectable CSF EBV-DNA load in two patients who had been occasionally treated with anti-herpetic therapy before and during the treatment of PCNSL, including acyclovir for genital herpes in one patient and gancyclovir for CMV retinitis in the other (17). A history of anti-herpetic therapy should be considered when interpreting the results of EBV-PCR. In addition, most previous reports on the high rate of the EBER expression in patients with AIDS-related PCNSL were conducted before or in the early HAART era (18), enrolling severely immunocompromised patients. Since the EBER expression is rare in immunocompetent PCNSL patients (19), our results of low EBER positivity indicate changes in the characteristics of ARL among HIV patients with relatively preserved immunity in the HAART era.

In this study, we found five patients with ARL-CNS(+) who were positive for EBV-DNA in the CSF but negative for the EBER expression in tissue. Notably, among all of the patients with EBER negative ARL-CNS(+), CSF EBV-DNA was detected only when plasma EBV-DNA was detectable, thus suggesting that plasma EBV-DNA transudation into CSF through the blood-brain barrier (BBB) is damaged by CNS involvement of ARL. The presence of plasma EBV-DNA among ARL patients is thought to reflect EBV replication, not in lymphoma tissue, but in other lymphatic tissues such as tonsil endothelial cells, under immunosuppression (20, 21). Although increased EBV activation may lead to ARL development, the increase in the EBV-DNA load in plasma and the EBER expression in tissue are not fully synchronized (20, 21). This may explain our finding of EBER-negative but CSF EBV-DNA positive ARL. Since our study is retrospective, the residue of specimens for EBER ISH was unavailable in 25%of the patients with CNS involvement. Further studies are needed to understand the role of CSF EBV-DNA measurement in the context of EBER-negative ARL.

Conclusion

The EBV-DNA load in the CSF is a marker of CNS involvement of ARL, with 200 copies/mL being a cut off level for the diagnosis of PCNSL and the identification of CNS involvement in patients with systemic ARL. Identifying EBV-DNA may help to differentiate the CNS lesions of ARL from other disorders.

The authors state that they have no Conflict of Interest (COI).

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CASE REPORT

Combination of high-dose dexamethasone and antiretroviral therapy rapidly improved and induced long-term remission of HIV-related thrombocytopenic purpura

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Abstract We present a case of HIV-related thrombocytopenic purpura (HIV-ITP) successfully treated with high-dose dexamethasone and antiretroviral therapy (ART). Although high-dose dexamethasone is regarded as the first-line therapy in adult patients with non-HIV ITP, there is limited information on treatment of HIV-ITP and long-term prednisone therapy is considered the standard therapy. High-dose dexamethasone is preferable to conventional long-term prednisone therapy, because of fewer side effects mainly due to shorter steroid use. The ART helps achieve long-term remission for HIV-ITP, although this therapy lacks an immediate effect. In our patient, administration of high-dose dexamethasone resulted in rapid rise in platelet count and ART maintained long-term remission of HIV-ITP. The combination therapy is potentially suitable strategy for the treatment of patients with HIV-ITP and severe thrombocytopenia or bleeding.

Keywords HIV-related immune thrombocytopenic purpura · High-dose dexamethasone · Antiretroviral therapy · HIV-1 infection

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Introduction

HIV-related thrombocytopenic purpura (HIV-ITP) is the most common cause of low platelet count encountered in patients with HIV-1 infection [1]. It is similar to classic immune thrombocytopenic purpura (ITP) in non-HIV patients, and long-term steroid therapy is regarded the standard treatment [2]. High-dose dexamethasone (HD-DXM) is effective in non-HIV ITP [3–5], however, little is known about its effectiveness in HIV-ITP [6, 7]. We describe a 72-year-old man who presented with HIV-ITP and was effectively treated with HD-DXM combined with antiretroviral therapy (ART).

Case report

A 72-year-old Japanese man was admitted to our hospital with thrombocytopenia. The patient had been diagnosed with HIV-1 infection 10 years earlier and ART was initiated 3 months after the diagnosis. However, adherence to therapy was poor, and the platelet count tended to decrease at times of high HIV-1 RNA viral load during poor adherence. Three months before admission, ART was changed to once-daily ritonavir-boosted darunavir (DRV/r) plus tenofovir/emtricitabine (TDF/FTC) to enhance adherence to therapy. Although repeated HIV-1 resistance testing showed no major mutation, HIV-1 RNA viral load was >1,000 copies/ml over several months. Apart from ART, there was no change in his medications and he had not had any infections during 6 months before admission. On admission, platelet count was 20,000/μl and CD4 count was 168/μl. The patient was alert and oriented with body temperature of 36.2 °C. Physical examination showed no signs of bleeding (e.g., no petechiae, purpura, or mucosal

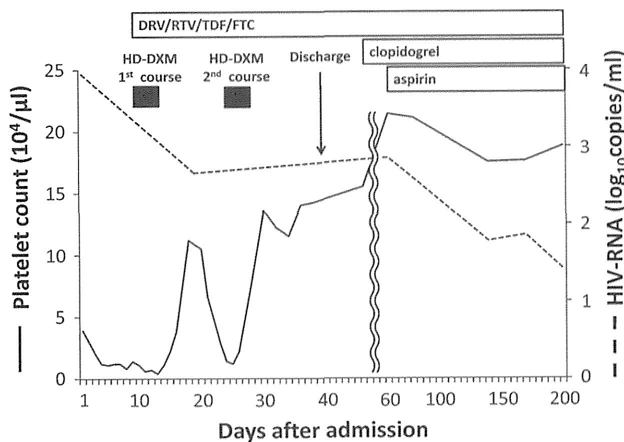

Fig. 1 Clinical course during hospitalization. *DRV* darunavir, *RTV* ritonavir, *TDF* tenofovir, *FTC* emtricitabine, *HD-DXM* high-dose dexamethasone

bleeding). To rule out drug-induced thrombocytopenia, ART, together with clopidogrel and aspirin, which had been administered for years, were discontinued on admission. Although platelet transfusion was initiated for a couple of days, no change in platelet count was noted. Bone marrow examination on day 5 showed hypocellularity with a low number of megakaryocytes. No histopathological findings specific to myelodysplastic syndrome or leukemia were noted. On day 10, the patient developed intermittent epistaxis with a platelet count of 4,000/µl. On that day, a four-day course of orally administered HD-DXM of 40 mg/day was initiated, and ART with DRV/r plus TDF/FTC was reinitiated. The platelet count increased to 66,000/µl on day 10 after the above treatment, but it decreased to 12,000/µl on day 14. A second course of HD-DXM of 40 mg/day was initiated. The platelet count improved to 115,000/µl on day 10 after the second course, and 142,000/µl on day 15. Based on such improvement, no third course was considered necessary. The patient was discharged on day 39 from admission. No adverse event of dexamethasone was observed. The platelet count remained stable after discharge despite the re-initiation of clopidogrel and aspirin (Fig. 1). Three months after re-initiation of ART and thereafter, the HIV-1 viral load was suppressed to <100 copies/ml with good medication adherence. The patient experienced no relapses of HIV-ITP for 9 months.

Discussion

We reported here a patient with HIV-ITP who was treated successfully with a combination with two courses of HD-DXM and ART. The ITP likely relapsed when the platelet count diminished to <90,000/µl on day 10 after the first course of HD-DXM [3], thus justifying the second course of HD-DXM. No additional courses were provided once the platelet count was above 90,000/µl on day 10 after the second course. That platelet count remained stable after re-initiating clopidogrel and aspirin negated any drug-induced thrombocytopenia. To our knowledge, this is the first case describing the use and effectiveness of the combination of HD-DXM and ART in the treatment of HIV-ITP.

In adult patients with non-HIV ITP, HD-DXM is preferred to conventional long-term prednisone [2], because of fewer adverse events, mainly due to the shorter term of steroid administration. The major side effect of steroid is immunosuppression, and it is important to avoid such complication, especially in immunocompromised hosts, such as HIV-1 infected patients. For the treatment of HIV-ITP, HD-DXM is probably as effective as in non-HIV ITP, because both diseases are considered to have a similar etiology [8]. Although about one-fifth of non-HIV ITP patients on HD-DXM treatment relapse by 8 months after treatment [5], the use of ART in patients with HIV-ITP can maintain long-term remission despite the lack of an immediate effect [9, 10]. In our patient, HD-DXM was applied when the clinical condition was severe with bleeding and thrombocytopenia, and it resulted in rapid improvement in platelet count. Thereafter, administration of ART resulted in suppression of viral load, which probably promoted long-term remission of HIV-ITP.

Notably, HD-DXM is cost-effective, compared to other treatments for ITP, such as immunoglobulin or rituximab. Although further studies are needed to confirm the efficacy and safety of the combination therapy, HD-DXM and ART is potentially suitable for treatment of HIV-ITP patients with severe thrombocytopenia or bleeding.

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Conflict of interest All authors declare no conflict of interest.

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Restriction fragment mass polymorphism (RFMP) analysis based on MALDI-TOF mass spectrometry for detecting antiretroviral resistance in HIV-1 infected patients

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Abstract

Viral genotype assessment is important for effective clinical management of HIV-1 infected patients, especially when access and/or adherence to antiretroviral treatment is reduced. In this study, we describe development of a matrix-assisted laser desorption/ionization-time of flight mass spectrometry-based viral genotyping assay, termed restriction fragment mass polymorphism (RFMP). This assay is suitable for sensitive, specific and high-throughput detection of multiple drug-resistant HIV-1 variants. One hundred serum samples from 60 HIV-1-infected patients previously exposed to nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs) and protease inhibitors (PIs) were analysed for the presence of drug-resistant viruses using the RFMP and direct sequencing assays. Probit analysis predicted a detection limit of 223.02 copies/mL for the RFMP assay and 1268.11 copies/mL for the direct sequencing assays using HIV-1 RNA Positive Quality Control Series. The concordance rates between the RFMP and direct sequencing assays for the examined codons were 97% (K65R), 97% (T69Ins/D), 97% (L74VI), 97% (K103N), 96% (V106AM), 97% (Q151M), 97% (Y181C), 97% (M184VI) and 94% (T215YF) in the reverse transcriptase coding region, and 100% (D30N), 100% (M46I), 100% (G48V), 100% (I50V), 100% (I54LS), 99% (V82A), 99% (I84V) and 100% (L90M) in the protease coding region. Defined mixtures were consistently and accurately identified by RFMP at 5% relative concentration of mutant to wild-type virus while at 20% or greater by direct sequencing. The RFMP assay based on mass spectrometry proved to be sensitive, accurate and reliable for monitoring the emergence and early detection of HIV-1 genotypic variants that lead to drug resistance.

Keywords: Drug, HIV-1, MALDI-TOF, resistance mutation, RFMP
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Introduction

Highly active antiretroviral therapy (HAART) can dramatically suppress HIV-1 replication, improve immunological response and extend a patient's lifespan. However, less than excellent adherence to HAART or conditions that result in reduced treatment efficacy leads to a higher risk of the emergence of antiretroviral (ARV) drug-resistant viral strains, which eventually leads to increased viral loads, poor immunological response and eventually treatment failure [1]. Especially, women who have received single-dose nevirapine to prevent mother-to-child HIV-1 transmission are at increased risk of virological failure as a result of the replication of low-abundance nevirapine-resistant variants when treated with a subsequent nevirapine-containing regimen [2]. Of importance in the effective management of HIV-1 infections is the timely and efficient detection of

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