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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

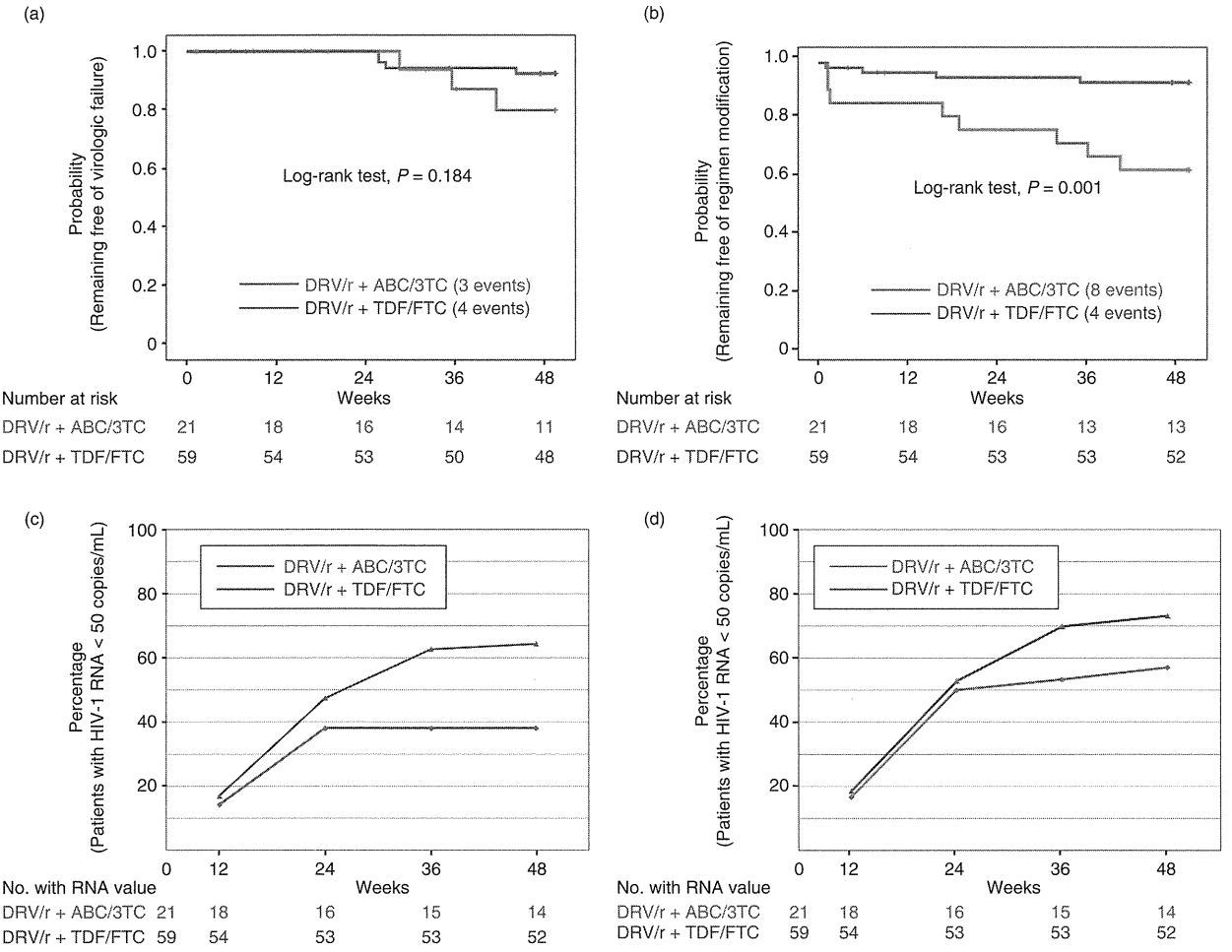

Fig. 1. Efficacy and tolerability results over 48 weeks. (a) Time to protocol-defined virologic failure. (b) Time to tolerability endpoint, defined as first change in treatment regimen. Percentage of patients with HIV-1 RNA less than 50 copies/ml at weeks 12, 24, 36, and 48, regardless of previous virologic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

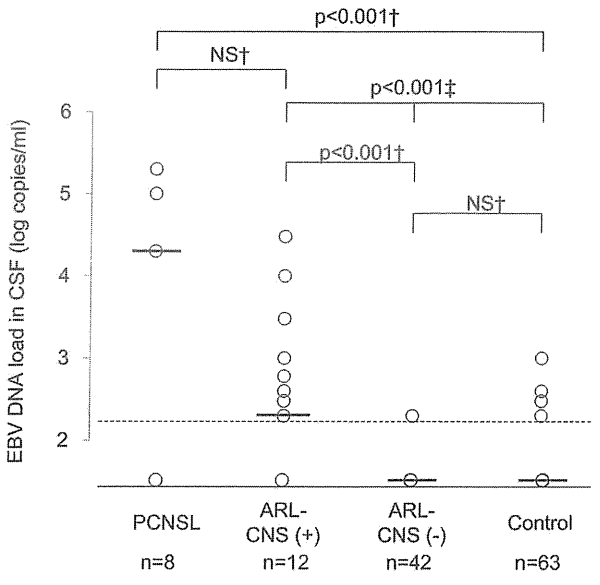

Figure 1. The EBV-DNA loads in the cerebrospinal fluid (CSF) of the patients with AIDS-related lymphoma and the control subjects. PCNSL: primary CNS lymphoma, ARL-CNS (+): systemic AIDS-related lymphoma with CNS involvement, ARL-CNS (-): systemic AIDS-related lymphoma without CNS involvement, NS: not 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.

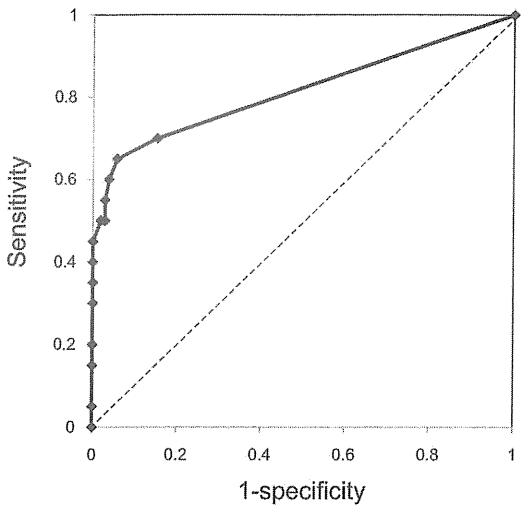

Figure 2. Receiver operating characteristic (ROC) curve for the cutoff values of the EBV-DNA load in the cerebrospinal fluid for the diagnosis of CNS involvement of systemic AIDS-related lymphoma. The dotted line is the reference line. The area under the ROC curve was 0.856 (95% confidence interval, 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

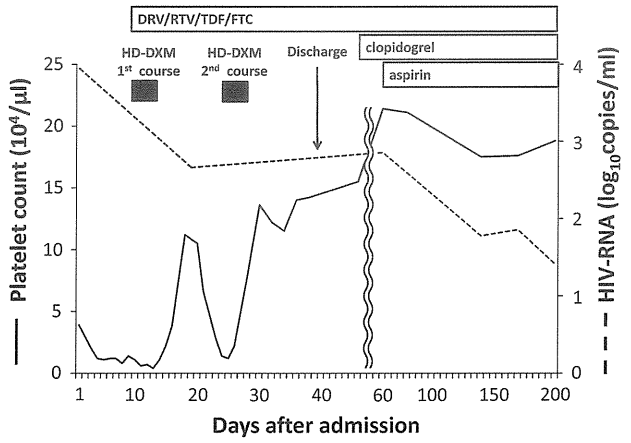

Fig. 1 Clinical course during hospitalization. *DRV* darunavir, *RTV* ritonavir, *TDF* tenofovir, *FTC* emtricitabine, *HD-DXM* high-dose dexamethasone

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

drug-resistant viral strains and their specific mutations in a patient's clinical samples.

The guidelines for use of ARV drugs in HIV-1-infected adults and adolescents established by the US Department of Health and Human Services (DHHS) (http://www.aidsinfo.nih.gov/guidelines) recommend monitoring viral genotypic changes in patient samples and use of this information to determine which therapeutic regimens are most appropriate for the specific patient [3].

The restriction fragment mass polymorphism (RFMP) method is based on amplification and mass detection of oligonucleotides excised by type-IIS restriction enzyme digestion, using matrix-assisted laser desorption and ionization time of flight mass spectrometry (MALDI-TOF MS). RFMP-based drug-resistance testing and genotyping has been shown to be a sensitive, accurate and reliable method for clinical utility in many fields [4–12]. Especially important is that RFMP enables sensitive detection of mutations without population-based cloning and subsequent sequencing analysis [6].

In this study, we applied the RFMP assay for detection of mutations in the coding sequences for reverse transcriptase (RT) and protease (PR) of HIV-1 that engender resistance to nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs) and protease inhibitors (PIs). Compared with direct sequencing, RFMP is shown to be a sensitive and reliable method for genotypic testing of drug-resistance mutations in HIV-1 infected patients.

Materials and Methods

Specimens

A total of 100 plasma samples were collected from 60 HIV-1 infected patients who had received HAART (including NRTIs, NNRTIs and PIs) at the AIDS Clinical Center, National Center for Global Health and Medicine, Japan, between 1999 and 2009. Written informed consent was obtained from each participant, and the experimental protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki, as reflected in *a priori* approval (NCGM-H22-938) by the Ethics Committee of the AIDS Clinical Center, National Center for Global Health and Medicine, Japan. The demographic characteristics are summarized in Table 1.

TABLE 1. Demographic characteristics of 60 HIV-1-infected patients

Characteristic	Value
Mean age in years (range)	42 (22–67)
No. male (%)	53 (88)
No. female (%)	7 (12)
Race (%)	
Asian	58 (97)
African	2 (3)
Risk factor for HIV (% of patients)	
Heterosexual	14 (23)
Homosexual	27 (45)
Haemophilia (infected blood products)	19 (32)
CDC clinical stage (%)	
A1/A2/A3	0/0/0
B1/B2/B3	17/23/13
C1/C2/C3	5/12/20
Unknown	10
Mean CD4 cell count (No. of cells/uL [range])	320 (12–759)
No. of unknown (%)	3 (5)
Mean HIV-1 RNA (No. of RNA copies/mL [range])	43 000 (50–1 200 000)
No. of unknown (%)	6 (10)
History of actual treatment (No. of patients)	
With NRTI	2
With NRTI plus NNRTI	23
With NRTI plus PI	21
With NNRTI plus PI	1
With NRTI plus NNRTI plus PI	33
With NRTI plus PI plus INI	1
With NRTI plus INI	2
With NRTI plus PI plus INI plus FI	1
With NRTI plus NNRTI plus PI plus INI plus FI	1
Interruption	15

NRTI, nucleoside reverse transcriptase inhibitor; lamivudine, abacavir, emtricitabine, tenofovir, stavudine, didanosine, zidovudine; NNRTI, non-nucleoside reverse transcriptase inhibitor; efavirenz, nevirapine; PI, protease inhibitor; atazanavir, ritonavir, lopinavir, darunavir, fosamprenavir, amprenavir, nelfinavir; INI, integrase inhibitor; raltegravir; FI, fusion inhibitor; enfuvirtide.

HIV-1 performance panels

To assess the limit of detection ability of the RFMP assay, the HIV-1 RNA Positive Quality Control Series (ACCURUN® 315) obtained from SeraCare Life Sciences (Milford, MA, USA) was used to measure viral load in HIV-1 performance panels.

Construction of recombinant HIV-1 clones

Recombinant infectious HIV-1 clones with various mutations in the RT region were constructed using site-directed mutagenesis. Briefly, the mutations were introduced into the *Xma*I-*Nhe*I fragment (759 bp) of pTZNX1, which encodes Gly-15 to Ala-267 of HIV-1 RT (strain BH 10), by oligonucleotide-based mutagenesis [13]. The *Xma*I-*Nhe*I fragment was inserted into a pNL4-3-based plasmid, generating various molecular clones with the desired mutations. Each molecular clone (10 /mL as DNA) was transfected into human 293T cells (4×10^5 cells/100-mm-diameter dish) with Fugene transfection reagent (Roche Diagnosis, Basel, Switzerland). After 48 h, culture supernatants were harvested and stored at −80°C until use. Viral loads were determined using the COBAS® Amplicor HIV-1 Monitor Test, v1.5.

RNA extraction and cDNA amplification

HIV-1 RNA was extracted from 200 μL of plasma using the High Pure Viral RNA Kit (Roche Diagnostics, Mannheim, Germany) according to manufacturer's instructions. Purified viral RNA was dissolved in 50 μL elution buffer (nuclease-free, sterile, double distilled water). cDNA was synthesized using only the reverse transcription step component of the RNA PCR kit (TaKaRa, Otsu, Japan).

RFMP assay

PCR reactions were performed in 25 μL reaction mixtures containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 0.2 mM deoxynucleoside triphosphates (dNTPs), 10 pmol of primers and 0.4 U of Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA). The initial denaturing phase of 5 min at 94°C was followed by a 35-cycle amplification phase containing a denaturation step at 94°C for 15 s, annealing step at 50°C for 15 s and elongation step at 72°C for 15 s, and completed with a final extension phase at 72°C for 5 min. For the RFMP analysis of codons 65, 69, 74, 103, 106, 151, 181, 184 and 215 in the HIV-1 RT region and codons 30, 46, 48, 50, 54, 82, 84 and 90 in the HIV-1 PR region, each of the forward and reverse primers contained the viral target sequence and the *Fok*I recognition sequence ggatg (Table S1). Restriction enzyme digests were performed by mixing the PCR reaction with 10 μL of buffer (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate and 1 mM dithiothreitol) and 1 U of *Fok*I enzyme (New England Biolabs, Beverly, MA, USA). The reaction mixtures were incubated at 37°C for 2 h. Subsequently, the digest desalting and mass analysis were performed as described previously [14].

MALDI-TOF instrumentation and calibration

Mass spectra were acquired on a Biflex IV linear MALDI-TOF MS (Bruker Daltonics) workstation equipped with a 337 nm nitrogen laser and a nominal ion flight path length of 1.25 m. The samples were analyzed in the negative-ion mode with a total acceleration voltage of 20 kV, extraction voltage of 18.25 kV, laser attenuation of 55, and delayed extraction of long time delay mode. Typically, time-of-flight data from 20 to 50 individual laser pulses were recorded and averaged on a transient digitizer with a time base of 2 ns and delay of 24 000 ns, after which the averaged spectra were automatically converted to mass by the accompanying data-processing software. Using these settings the instrument typically provided mass accuracy of 40–80 ppm (10^{-6}), mass resolution of 1500–2000 and sensitivity of 10–50 fmol in the 2- to 6-kDa mass range for oligonucleotides. Oligonucleotide standards of 6mer (5′-ACGTAC-3′; 1762.2 Da) and 16mer (5′-AC GTACGTACGTACGT-3′; 4881.2 Da) with no terminal phosphate were used for mass calibration of the instrument. The presence of metal cations produces salt adducts, leading to reduced resolution and low sensitivity, so C18 reverse phase micro-column chromatography was used for desalting oligonucleotides. Non-homogeneous crystallization is obtained with the classic dried droplet preparation, and a search for a 'sweet spot' is required. Re-crystallization of sample DNAs on matrix-prespotted anchorchip plates allowed robust formation of small single crystals.

Direct sequencing assay

To amplify the HIV-1 RT and PR regions for analysis by direct sequencing, PCR was performed with the following primers: 5′-AACAATGGCCATTGACAGAAGAAA-3′ (2614–2637 bp of HXB2), 5′-CTGTATGTCATTGACAGTCCAGCT-3′ (3299–3323 bp of HXB2) for the RT region and 5′-CTTCCCTCA GATCACTCTTTGGCAA-3′ (2248–2273 bp of HXB2), 5′-AGGGCTAATGGGAAAATTTAAAGT-3′ (2238–2561 bp of HXB2) for the PR region. PCR products were sequenced using the BigDye Terminator (version 3.1) Cycle Sequencing kit and an ABI PRISM 310 Analyzer (Applied Biosystems, Foster City, CA, USA).

Statistics

A limit of detection test was performed by probit analysis to compare sensitivity between the RFMP and direct sequencing assays using the statistical package SAS (version 8; SAS Institute Inc., Cary, NC, USA).

Results

RFMP assay strategy

The RFMP assay is based on mass spectrometric analysis of small DNA fragments that include sites of mutation (Fig. S1). The first step requires PCR amplification with forward and reverse primers that introduce the *Fok*I enzyme site, ggatg (Table S1). The diagnostic fragments released by enzymatic digestion consist of various sizes from 8 nt oligomers to 14 nt oligomers for nine codons in the RT region and eight codons in the PR region, leading to facile identification of sequence variation by mass spectrum analysis. Genotypic analysis of mutations at codons 65, 69, 74, 103, 106, 151, 181, 184 and 215 in the RT region and codons 30, 46, 48, 50, 54, 54, 82, 84 and 90 in the PR region, as assessed by the RFMP assay, was determined for 100 plasma samples. The RFMP results showed distinct peaks relevant to each codon, with the mass values for each diagnostic fragment being exactly as predicted (Supplementary Material Table S2).

Estimation of limit of detection and ability to detect mixed genotype populations

The detection limit was estimated using replicates of each of nine dilutions of HIV-1 RNA Positive Quality Control Series (ACCURUN® 315) material ranging between 10 and 5000 copies/ml. Analysis of various calibrated HIV-1 RNA dilution series determined the lower detection limit to be 223.02 copies/mL for the RFMP and 1268.11 copies/mL for the direct sequencing assays by probit analysis. The probit analysis predicts a 95% CI: 132.64–693.00 for the RFMP and 863.09–3656.80 for the direct sequencing assays (Table 2).

TABLE 2. Limit of detection of the RFMP and direct sequencing assays

		RFMP		Direct sequencing	
HIV-1 RNA copies/ml	No. tested	No. detected	Per cent detected	No. detected	Per cent detected
5000	10	10	100%	10	100%
2500	10	10	100%	10	100%
1000	10	10	100%	9	90%
500	10	10	100%	4	40%
250	10	10	100%	1	10%
100	10	8	80%	0	0%
50	10	3	30%	0	0%
25	10	1	10%	0	0%
10	10	1	10%	0	0%
Limit of detection		223.02 copies/mL (95% CI, 132.64–693.00)		1268.11 copies/mL (95% CI, 863.09–3656.80)	

Defined dilutions of HIV-1 RNA Positive Quality Control Series were made from 10 copies to 5000 copies/mL and limit of detection abilities were calculated by probit analysis at a 95% detection level.

To evaluate the ability of the RFMP assay to determine small amounts of mutant virus in mixed populations, assays were performed with recombinant HIV-1 clones composed of different ratios of wild-type (K103 in the RT region) and mutant genotypes (N103 in the RT region). Defined mixtures were prepared with the following percentages of K103N mutant virus in the total virus population: 100%, 50%, 20%, 10%, 5% and 1%. The K103N mutant virus could be detected in concentrations as low as 5% of the total virus by RFMP, whereas direct sequencing assays were able to detect mutant virus only when present in 20% or more of the total virus population (Fig. 1).

Comparison of RFMP with direct sequencing analyses

All 100 clinical samples from 60 patients were analysed by the RFMP and direct sequencing assays for the presence of drug resistance-related mutations: nine codons in the RT region and eight codons in the PR region of the HIV-1 *pol* gene (a total of 17 codons).

The overall concordance rates between RFMP and sequencing assays were excellent, irrespective of PR and RT regions (Table 3). Concordance rates in the RT region were 97% (97/100) at codons 65, 69, 74, 103, 151, 181 and 184, 96% (96/100) at codon 106, and 94% (94/100) at codon 215 (Fig. 2a).

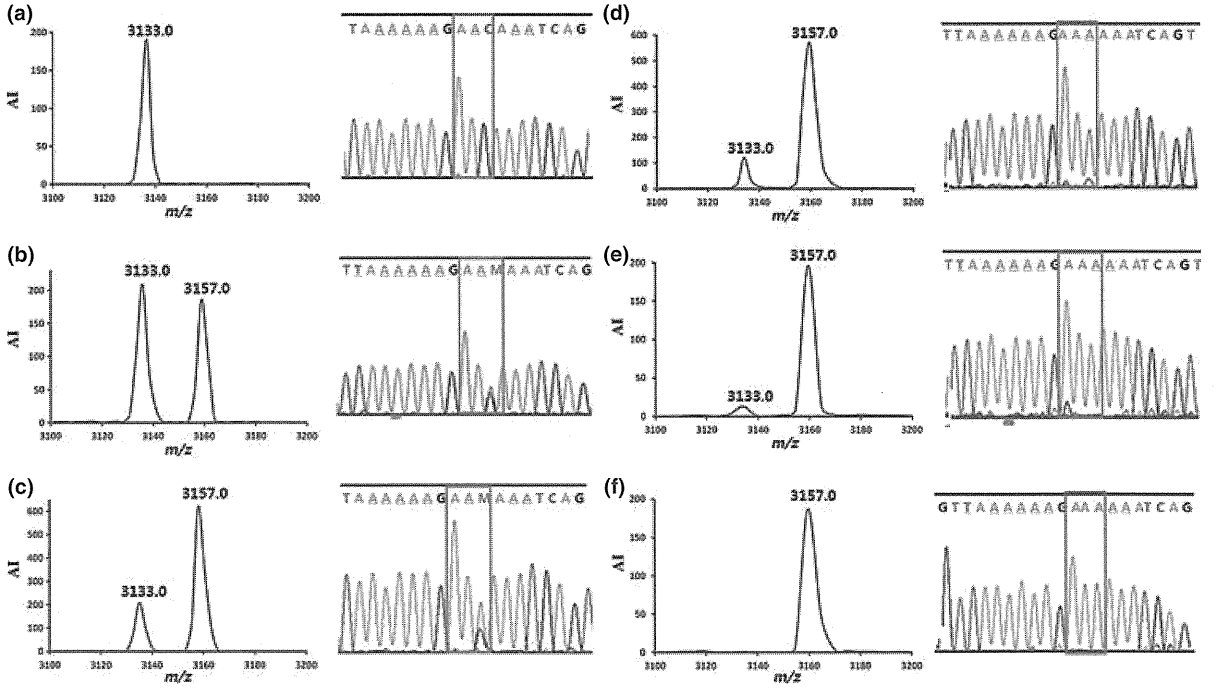

FIG. 1. Evaluation of the sensitivity of the RFMP assay for detection of minor amounts of virus with a defined mixture of K103N. The MALDI-TOF MS spectra and direct sequencing chromatograms shown are representative of experiments repeated three times using mixed populations of wild-type (K103) and NNRTI mutant (N103). The wild-type plasmids were mixed with mutant type at various ratios as follows: (a) 100%, (b) 50%, (c) 20%, (d) 10%, (e) 5% and (f) 1%. Molecular masses of 3133.0 and 3157.0 correspond to N103 and K103, respectively. AI is absolute intensity; m/z is mass-to-charge ratio.

TABLE 3. Comparison of the results obtained by the RFMP and direct sequencing assays in 100 clinical specimens

				Compatible (*n*)		Amino acid	
pol gene	Codon	Discordant (*n*)	Concordant (*n*)	RFMP only	Direct sequencing only	RFMP	Direct sequencing
RT	65	3	97	–	–		
	69	3	97	–	–		
	74	3	97	–	–		
	103	3	97	–	–		
	106	3	96	1	–	V/A	V
	151	3	97	–	–		
	181	3	97	–	–		
	184	3	97	–	–		
	215	3	94	3	–	T/F	T/F or I/S or T/I/F
						T/Y	T/Y or N/S or T/S/Y
						T/Y	T/Y or N/S or T/S/Y
PR	30	–	100	–	–		
	46	–	100	–	–		
	48	–	100	–	–		
	50	–	100	–	–		
	54	–	100	–	–		
	82	–	99	1	–	V/A	V
	84	–	99	1	–	I/V	V
	90	–	100	–	–		

RT, reverse transcriptase; PR, protease.

Similarly, concordance rates in the PR region were 100% (100/100) at codons 30, 46, 48, 50, 54 and 90, and 99% (99/100) at codons 82 and 84 in the PR region (Fig. 2b). Both assays showed identical base substitution and amino acid composition in these positions. Rate of compatible cases were observed 1% (1/100) at codons 106, and 3% (3/100) at codon 215 in the RT region and 1% (1/100) at codons 82 and 84 in the PR region, respectively. Three samples (mixed-type) at codon 215 containing double mutations in a single codon were identified only by RFMP, as a result of the inability of the direct sequencing assay to determine the variants present in the clinical samples. As shown in Fig 2(c), 215T/Y mixtures detected by RFMP could be scored as 215T (ACC) plus 215Y (TAC), or 215N (AAC) plus 215S (TCC), or 215T (ACC) plus 215S (TCC) plus 215Y (TAC), by direct sequencing. A compatible single nucleotide mixture at one position was observed in three samples at three codons (codon 106 in the RT; codon 82, 84 in the PR), respectively. Of these, the RFMP assay detected more mixed samples than the direct sequencing assay. The details of mixtures detected by both assays are shown in Table 3. Discordances between the two assays occurred for three samples at RT region codons, which had undetectable viral loads; the correct viral genotypes were identified only by RFMP assay.

Discussion

ARV drug-resistance is a major obstacle in the effective clinical management of HIV-1-infected patients [3,15] and therapeutic strategies must maximize the early detection of drug resistance mutations. Having a sensitive genotyping assay that can detect with high accuracy and reliability, drug-resistance mutations that emerge during HAART can be very important for the optimization of ARV regimens, improvement of patient treatment and outcome of therapy. The RFMP assay has been demonstrated to be a sensitive, accurate and reliable method for genotyping and detecting drug-resistance mutations in several viruses, including hepatitis and papillomavirus [4,6,8–12].

In the present study, we validated use of the MALDI-TOF MS-based RFMP assay to detect oligonucleotides containing 8–14 nucleotides for codons implicated with ARV drug-resistance mutations. Specifically, we established successful detection at codons 65, 69, 74, 103, 106, 151, 181, 184 and 215 in the HIV-1 RT coding region, and 30, 46, 48, 50, 54, 82, 84 and 90 in the HIV-1 PR coding region. These codons address resistance to all approved NRTI and NNRTI inhibitors [16–18]: mutation at RT codon 65 (K65R) confers resistance to tenofovir, didanosine and abacavir; RT mutation L74V confers resistance to didanosine and abacavir; the K103N RT mutation engenders resistance to the NNRTIs efavirenz and nevirapine; the Q151M RT mutation causes resistance to AZT, D4T, didenosine and abacavir through the decreased incorporation mechanism; Y181C causes resistance to nevirapine, etravirine and rilpivirine; M184V/I confers resistance to 3TC and FTC, and also affects resistance to rilpivirine; and finally, T215Y causes resistance to AZT and D4T through the excision mechanism. In addition, mutations at the 30, 46, 48, 50, 54, 82, 84 and 90 sites of the HIV-1 PR coding region cause resistance to all known protease inhibitors: specifically, D30N causes resistance to nelfinavir; M46I/L causes resistance to nelfinavir and indinavir; G48V/M causes resistance to atazanavir, nelfinavir and saquinavir; I50L/V causes resistance to atazanavir, darunavir, fosamprenavir and lopinavir; and mutations at

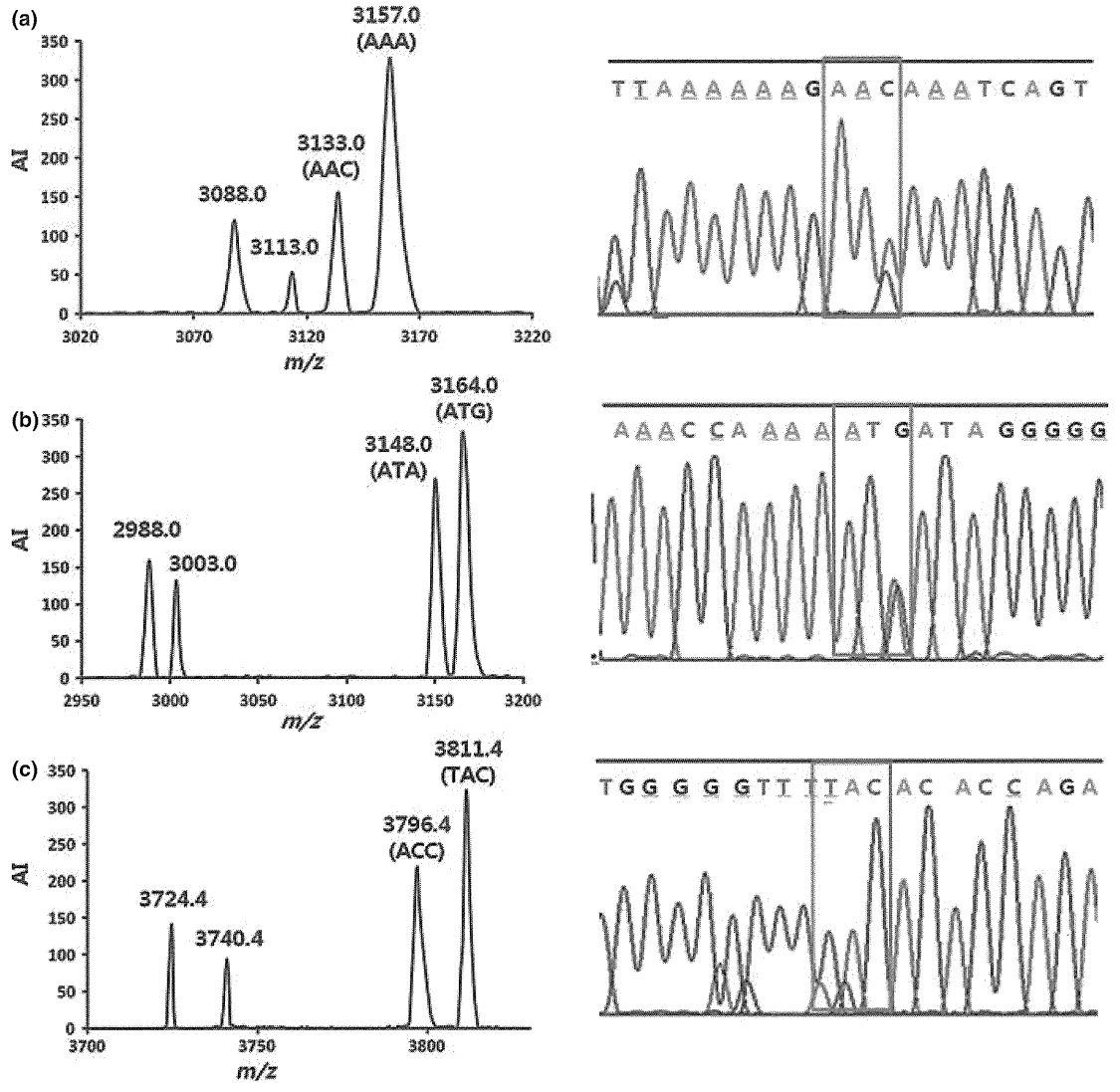

FIG. 2. Comparison of the RFMP and direct sequencing assays for detection of mixed genotypes. Sera were taken from patients infected with HIV-1 carrying ARV drug-resistant mutations and examined by the RFMP and sequencing assays. (a) For codon 103, molecular masses of 3088.0/3157.0 and 3113.0/3133.0 represent Lys (AAA) and Asn (AAC), respectively. (b) For codon 46, molecular masses of 2988.0/3164.0 and 3003.0/3148.0 represent Met (ATG) and Ile (ATA), respectively. (c) For codon 215, molecular masses of 3740.4/3796.4 and 3724.4/3811.4 represent Thr (ACC) and Tyr (TAC), respectively. Each codon was indicated by a red box in the sequencing chromatogram. AI is absolute intensity; m/z is mass-to-charge ratio.

residues 54, 82, 84 and 90 cause resistance to all protease inhibitors to a varying extent (http://hivdb.stanford.edu/DR/PIResiNote.html).

The detection limit of the RFMP assay was determined to be 223.02 copies/mL and able to identify a minority mutant at a concentration as low as 5% of the circulating mixed populations, whereas the detection limit of the direct sequencing method was 1268.11 copies/mL and able to detect variants only when present at >20% of the total population (Table 2 and Fig. 1). A clear correlation was observed between peak ratios and relative genotype concentration of mixed populations.

The performance of the RFMP assay in detecting mutations related to ARV drug-resistance was compared with direct sequencing assays for 100 clinical samples from 60 HIV-1-infected patients who experienced HAART therapy. The RFMP assay successfully identified genotypic changes at all 17 codons tested in clinical samples. Compared with direct sequencing, the RFMP assay exhibited 96.6% concordance in the RT region and 99.8% concordance in the PR region (Table 3 and Fig 2). The PR data were not significantly more concordant than the RT data (the difference was only two cases). The reason for the slight difference is not clear, thus further investigation is required with more samples. Notably, the RFMP assay

outperformed direct sequencing for the detection of single and double mutations in compatible samples. The RFMP assay detected 1% (1/100) more mutant viruses in codons 106, 82 and 84 and 3% (3/100) more mutant viruses in codon 215. All discordances between the two assays were due to the inability of the direct sequencing assay to identify the residues at nine RT codons in three patients. Hence, the discrepancy among the two assays may be due to a lower sensitivity of direct sequencing.

HAART can clearly extend the life expectancy of HIV-1 patients. However, as adherence is usually imperfect, continuous ARV drug-resistance testing can be an important management tool. There are two major methods of assessing ARV drug-resistance: phenotypic assays and genotypic assays that provide complementary information and be preferable to the other [19,20]. Current treatment guidelines define as treatment failure the detection of more than 200 copies/mL of HIV-1 RNA [3]. However, existing genotypic assays, such as direct sequencing and the reverse hybridization-based assay, do not reliably detect fewer than 1000 copies/ml of HIV-1 RNA, nor do they enable detection of sequence variants present at <20% of minority variants in mixed populations [21,22]. This performance does not allow facile interpretation of ratios between multiple virus mixtures, especially when a double mutation is present in a single codon. While the reverse hybridization-based assay has somewhat higher sensitivity than direct sequencing it gives rise to false-positive and false-negative results more frequently than direct sequencing [21–26].

With the advent of Next Generation Sequencing methods it is possible to detect, by 454 pyrosequencing or an Illumina Genome Analyzer, minor viral variants whose prevalence is <1%. 454 pyrosequencing, also called ultra-deep pyrosequencing (UDPS), relies on fixing nebulized and adapter-ligated DNA fragments to small DNA-capture beads in a water-in-oil emulsion. The DNA fixed to these beads is then amplified by PCR. An advantage of UDPS in the case of viral genomic analysis is that it yields long sequence information for each sample (average ~800 bases). However, UDPS has some technical challenges. A major limitation of the UDPS relates to resolution of homopolymer-containing DNA segments, such as AAA and GGG [27]. Because there is no terminating moiety preventing multiple consecutive incorporations at a given cycle, pyrosequencing relies on the magnitude of light emitted to determine the number of repetitive bases. This is prone to greater error than misincorporation. Hence, the dominant error type for the 454 platform is insertion-deletion, rather than substitution. The decrease in single read accuracy from 99.4% for test fragments to 96% for genomic libraries is primarily due to a lack of clonality in a fraction of the genomic templates in the emulsion [28]. Moreover, based on current list prices for the UDPS, the current cost for all the reagents, including the picotiter plate, library preparation kits and emulsion PCR kits, to perform a single experiment is $1000–7000 [29].

By combining the merits of unique assay chemistry and the mature nature of MALDI-TOF mass spectrometry, the RFMP assay can be used to screen for HIV drug-resistance mutations in a robust high-throughput manner (e.g. 96 samples can be analysed in 3 h with Bruker software (flexcontrol 3.0), which is faster than existing hybridization or sequencing-based methods). In terms of cost-effectiveness, the direct cost per test (reagents and labour) of the RFMP assay can be <$30, including viral DNA extraction, PCR, restriction digestion, desalting and matrix for mass analysis, which is cheaper than the sequencing or hybridization assays that are *c.* $50–100 per test. These costs do not include the equipment costs, which are slightly greater for the RFMP method. However, with the advent of many diagnostic assays operating on a mass spectrometer platform, such as clinical genotyping and microorganism identification, and the gradual spread of compact mass spectrometers into laboratories as medical devices (i.e. Bruker Microflex), the burden of the amortization cost should be substantially reduced [30]. A limitation of the RFMP assay is that it determines only molecular mass and is thus applicable only to known resistance mutations and may fail to detect DNA sequence changes that do not affect molecular mass. Moreover, the occurrence of a novel resistance mutation with sequence alterations that cause a deviation from the predicted molecular mass pattern should be addressed by periodic updating of mass patterns for unambiguous result interpretation from up-to-date HIV databases.

We demonstrate here that the mass spectrometry-based RFMP assay is highly sensitive and able to successfully detect HIV-1 carrying drug-resistant mutations that are present in <5% of the total virus population. Hence, this assay can be used for the efficient assessment of genotype dynamics of viral quasispecies. Therefore, this simple procedure can be easily adapted to a high-throughput format, should enable earlier detection of drug-resistant viruses and help elucidate mechanisms of HIV-1 resistance, as well as guide and optimize treatment decisions.

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Transparency Declaration

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