TABLE 1. CLINICAL CHARACTERISTICS OF PATIENTS WITH AND WITHOUT CMV-GID

	CMV-GID (n=12)	*Non CMV-GID (n=88)*	p *Value*
Age (IQR)	39 (36–46)	40 (37–51)	0.451
Male gender (%)	11 (91.7)	87 (98.9)	0.227
CD4 count (/μL) (IQR)	68.5 (28.8–123.3)	84 (38.3–151.0)	0.356
HIV viral load (log10/mL) (IQR)	4.58 (3.27–5.24)	2.84 (1.60–5.08)	0.084
History of HARRT (%)	3 (5.2)	55 (62.5)	0.016
MSM (%)	9 (75.0)	69 (78.4)	0.723
Positive CMV antigenemia (%)	9 (75.0)	18 (20.5)	<0.001
Epigastric pain (SD)	2.5 (2.1)	1.8 (1.3)	0.373
Heartburn (SD)	2.5 (1.5)	1.8 (1.3)	0.064
Nausea and vomiting (SD)	2.4 (1.7)	2.0 (1.5)	0.384
Odynophagia (SD)	2.1 (1.7)	1.7 (1.5)	0.481
Chronic diarrhea (SD)	2.3 (1.3)	1.8 (1.4)	0.078
Bloody stool (SD)	2.5 (2.0)	1.7 (1.5)	0.021

CD4 cell counts within 1 week and HIV-RNA viral load within 1 month were checked at the day of endoscopy. A positive result for real-time HIV RNA was defined as ≥40 copies/mL. History of HAART was collected from the medical records prior to endoscopy. Sexual behavior was defined as men who have sex with men (MSM) or heterosexual.

CMV, cytomegalovirus; GID, gastrointestinal disease; HAART, highly active antiretroviral therapy; IQR, interquartile range; MSM, men who have sex with men; SD, standard deviation.

study.[8–10,22–26] This difference could be explained by the fact that the current study focused on gastrointestinal disease, while previous studies included various CMV diseases such as retinitis, cholangitis, pneumonia, and encephalitis.[8–10,22–26] The diagnostic accuracy of CMV antigenemia may vary depending on the site and extent of organ/tissue involvement.

Identification of CMV cells in tissue samples obtained by endoscopic biopsy is considered the gold standard for the diagnosis of CMV-GID.[1,2,6] The endoscopic findings in CMV-GID include ulcer and mucosal inflammation;[16,17] however, physicians may not consider it necessary to take a biopsy in patients with only mucosal inflammation without ulceration. Even in cases of severe deep or bleeding ulcers, some physicians may hesitate to perform a biopsy. In such cases, no definite diagnosis of CMV-GID can be made. Our results suggest that the CMV antigenemia assay is to some extent useful for the diagnosis of CMV-GID in patients with endoscopic findings, especially when CMV positive cell counts are high. Considering the high specificity and high positive LR (5.5) of the positive CMV cell count ≥5, the use of this method before endoscopy could potentially avoid complications due to biopsy.

One limitation of this study was the single-center nature of the investigation. Significant differences in independent factors were not detected in the present study probably due to the small number of patients with CMV-GID. For example, we used gastrointestinal symptoms with score of 7 points on the Likert scale, but the differences in most symptoms between patients with or without CMV-GID did not reach statistical significance due to the small number of cases. Further studies based on larger population are needed. Another limitation is a selection bias related to the selection criteria applied in the present study: only patients who underwent endoscopy for such reasons as symptoms and screening were included in the study.

In conclusion, the CMV antigenemia assay showed relatively good sensitivity and specificity for the diagnosis of CMV-GID in patients with HIV infection. Furthermore, specificity and positive LR improved when the cutoff value of CMV cell count was increased from 1 to ≥5 positive cells per 300,000 granulocytes. Considering the high specificity of the test, the use of this method before endoscopy could potentially avoid complications due to biopsy.

TABLE 2. DIAGNOSTIC ACCURACY OF CMV ANTIGENEMIA ASSAY FOR CMV-GID USING DIFFERENT CUTOFF VALUES AND HISTORY OF HAART

	Sensitivity (95%CI)	*Specificity (95%CI)*	*PPV (95%CI)*	*NPV (95%CI)*	*LR+ (95%CI)*	*LR− (95%CI)*	*OR (95%CI)*
CMV antigenemia ≥1 positive cell	75.0% (42.8–94.5)	79.5% (69.6–87.4)	33.3% (16.5–54.0)	95.9% (8.5–99.1)	3.7 (2.2–6.2)	0.31 (0.11–0.84)	11.7 (3.1–44)
CMV antigenemia ≥5 positive cells	50.0% (21.1–78.9)	90.9% (82.9–96.0)	42.9% (17.7–71.1)	93% (85.4–97.4)	5.5 (2.3–13.1)	0.55 (0.31–0.97)	10.0 (2.7–37.1)
History of HAART							
Yes[a]	66.7% (9.4–99.2)	83.6% (71.2–92.2)	18.2% (2.3–51.8)	97.9% (88.7–99.9)	3.7 (2.2–6.2)	0.31 (0.11–0.84)	10.2 (1.2–NA)
No[a]	77.8% (40.0–97.2)	72.7% (54.5–86.7)	43.8% (19.8–70.1)	92.3% (74.9–99.1)	2.9 (1.5–5.5)	0.31 (0.88–1.1)	9.33 (1.79–NA)

[a]Cutoff value of ≥1 positive cell per 300,000 granulocytes was used in the analysis.

CMV, cytomegalovirus; HAART, highly active antiretroviral therapy; LR+, positive likelihood ratio; LR−, negative likelihood ratio; NPV, negative predictive value; OR, odds ratio; PPV, positive predictive value.

Acknowledgments

We are grateful to Hisae Kawashiro (Clinical Research Coordinator) for help with data collection. The authors thank all other clinical staff at the AIDS Clinical Center and all the staff of the endoscopy unit.

This work was supported by Grants-in Aid for AIDS research from the Japanese Ministry of Health, Labor, and Welfare (H23-AIDS-001), and the Global Center of Excellence Program (Global Education and Research Center Aiming at the Control of AIDS) from the Japanese Ministry of Education, Science, Sports and Culture.

Author Disclosure Statement

The other authors declare no conflict of interest.

References

1. Goodgame RW. Gastrointestinal cytomegalovirus disease. Ann Intern Med 1993;119:924–935.
2. Baroco AL, Oldfield EC. Gastrointestinal cytomegalovirus disease in the immunocompromised patient. Curr Gastroenterol Rep 2008;10:409–416.
3. Toogood GJ, Gillespie PH, Gujral S, et al. Cytomegalovirus infection and colonic perforation in renal transplant patients. Transpl Int 1996;9:248–251.
4. Almeida N, Romaozinho JM, Amaro P, Ferreira M, Cipriano MA, Leitao MC. Fatal mid-gastrointestinal bleeding by cytomegalovirus enteritis in an immunocompetent patient. Acta Gastroenterol Belg 2009;72:245–248.
5. Frank D, Raicht RF. Intestinal perforation associated with cytomegalovirus infection in patients with acquired immune deficiency syndrome. Am J Gastroenterol 1984;79:201–205.
6. Korkmaz M, Kunefeci G, Selcuk H, et al. The role of early colonoscopy in CMV colitis of transplant recipients. Transplant Proc 2005;37:3059–3060.
7. Skiest DJ, Crosby C. CMV pp65 antigen testing is of limited utility in the diagnosis of concomitant CMV disease in HIV-infected patients in the HAART era. J Clin Virol 2003;28:203–213.
8. Podzamczer D, Ferrer E, Garcia A, et al. pp65 antigenemia as a marker of future CMV disease and mortality in HIV-infected patients. Scand J Infect Dis 1997;29:223–227.
9. Bek B, Boeckh M, Lepenies J, et al. High-level sensitivity of quantitative pp65 cytomegalovirus (CMV) antigenemia assay for diagnosis of CMV disease in AIDS patients and follow-up. J Clin Microbiol 1996;34:457–459.
10. Wattanamano P, Clayton JL, Kopicko JJ, et al. Comparison of three assays for cytomegalovirus detection in AIDS patients at risk for retinitis. J Clin Microbiol 2000;38:727–732.
11. Svedlund J, Sjodin I, Dotevall G. GSRS—A clinical rating scale for gastrointestinal symptoms in patients with irritable bowel syndrome and peptic ulcer disease. Dig Dis Sci 1988; 33:129–134.
12. Revicki DA, Wood M, Wiklund I, Crawley J. Reliability and validity of the Gastrointestinal Symptom Rating Scale in patients with gastroesophageal reflux disease. Qual Life Res 1998;7:75–83.
13. Gondo H, Minematsu T, Harada M, et al. Cytomegalovirus (CMV) antigenaemia for rapid diagnosis and monitoring of CMV-associated disease after bone marrow transplantation. Br J Haematol 1994;86:130–137.
14. Kanda Y, Mineishi S, Saito T, et al. Pre-emptive therapy against cytomegalovirus (CMV) disease guided by CMV antigenemia assay after allogeneic hematopoietic stem cell transplantation: a single-center experience in Japan. Bone Marrow Transplant 2001;27:437–444.
15. Mori T, Okamoto S, Matsuoka S, et al. Risk-adapted preemptive therapy for cytomegalovirus disease in patients undergoing allogeneic bone marrow transplantation. Bone Marrow Transplant 2000;25:765–769.
16. Rene E, Marche C, Chevalier T, et al. Cytomegalovirus colitis in patients with acquired immunodeficiency syndrome. Dig Dis Sci 1988;33:741–750.
17. Ljungman P, Griffiths P, Paya C. Definitions of cytomegalovirus infection and disease in transplant recipients. Clin Infect Dis 2002;34:1094–1097.
18. Mori T, Mori S, Kanda Y, et al. Clinical significance of cytomegalovirus (CMV) antigenemia in the prediction and diagnosis of CMV gastrointestinal disease after allogeneic hematopoietic stem cell transplantation. Bone Marrow Transplant 2004;33:431–434.
19. Fica A, Cervera C, Perez N, et al. Immunohistochemically proven cytomegalovirus end-organ disease in solid organ transplant patients: Clinical features and usefulness of conventional diagnostic tests. Transpl Infect Dis 2007;9:203–210.
20. Jang EY, Park SY, Lee EJ, et al. Diagnostic performance of the cytomegalovirus (CMV) antigenemia assay in patients with CMV gastrointestinal disease. Clin Infect Dis 2009;48: e121–124.
21. Nagata N, Kobayakawa M, Shimbo T, et al. Diagnostic value of antigenemia assay for cytomegalovirus gastrointestinal disease in immunocompromised patients. World J Gastroenterol 2011;17:1185–1191.
22. Pannuti CS, Kallas EG, Muccioli C, et al. Cytomegalovirus antigenemia in acquired immunodeficiency syndrome patients with untreated cytomegalovirus retinitis. Am J Ophthalmol 1996;122:847–852.
23. Boivin G, Handfield J, Murray G, et al. Quantitation of cytomegalovirus (CMV) DNA in leukocytes of human immunodeficiency virus-infected subjects with and without CMV disease by using PCR and the SHARP Signal Detection System. J Clin Microbiol 1997;35:525–526.
24. Wetherill PE, Landry ML, Alcabes P, Friedland G. Use of a quantitative cytomegalovirus (CMV) antigenemia test in evaluating HIV+ patients with and without CMV disease. J Acquir Immune Defic Syndr Hum Retrovirol 1996;12: 33–37.
25. Salzberger B, Franzen C, Fatkenheuer G, et al. CMV-antigenemia in peripheral blood for the diagnosis of CMV disease in HIV-infected patients. J Acquir Immune Defic Syndr Hum Retrovirol 1996;11:365–369.
26. Pellegrin I, Garrigue I, Binquet C, et al. Evaluation of new quantitative assays for diagnosis and monitoring of cytomegalovirus disease in human immunodeficiency virus-positive patients. J Clin Microbiol 1999;37:3124–3132.

Address correspondence to:
Naoyoshi Nagata, M.D.
Department of Gastroenterology and Hepatology
National Center for Global Health and Medicine
1-21-1, Toyama, Shinjuku-ku
Tokyo 162-8655
Japan

E-mail: nnagata_ncgm@yahoo.co.jp

OPEN ACCESS Freely available online

PLOS | ONE

Pharmacokinetics of Rifabutin in Japanese HIV-Infected Patients with or without Antiretroviral Therapy

Junko Tanuma[1]*, Kazumi Sano[2], Katsuji Teruya[1], Koji Watanabe[1], Takahiro Aoki[1], Haruhito Honda[1], Hirohisa Yazaki[1], Kunihisa Tsukada[1], Hiroyuki Gatanaga[1], Yoshimi Kikuchi[1], Shinichi Oka[1]

1 AIDS Clinical Center, National Center for Global Health and Medicine, Tokyo, Japan, **2** Department of Drug Metabolism and Disposition, Meiji Pharmaceutical University, Tokyo, Japan

Abstract

Objective: Based on drug-drug interaction, dose reduction of rifabutin is recommended when co-administered with HIV protease inhibitors for human immunodeficiency virus (HIV)-associated mycobacterial infection. The aim of this study was to compare the pharmacokinetics of rifabutin administered at 300 mg/day alone to that at 150 mg every other day combined with lopinavir-ritonavir in Japanese patients with HIV/mycobacterium co-infection.

Methods: Plasma concentrations of rifabutin and its biologically active metabolite, 25-*O*-desacetyl rifabutin were measured in 16 cases with HIV-mycobacterial coinfection. Nine were treated with 300 mg/day rifabutin and 7 with 150 mg rifabutin every other day combined with lopinavir-ritonavir antiretroviral therapy (ART). Samples were collected at a median of 15 days (range, 5–63) of rifabutin use.

Results: The mean C_{max} and AUC_{0-24} of rifabutin in patients on rifabutin 150 mg every other day were 36% and 26% lower than on 300 mg/day rifabutin, while the mean C_{max} and AUC_{0-24} of 25–*O*-desacetyl rifabutin were 186% and 152% higher, respectively. The plasma concentrations of rifabutin plus its metabolite were similar between the groups within the first 24 hours, but it remained low during subsequent 24 to 48 hours under rifabutin 150 mg alternate day dosing.

Conclusion: Rifabutin dose of 150 mg every other day combined with lopinavir-ritonavir seems to be associated with lower exposure to rifabutin and its metabolite compared with rifabutin 300 mg/day alone in Japanese patients. Further studies are needed to establish the optimal rifabutin dose during ART. The results highlight the importance of monitoring rifabutin plasma concentration during ART.

Trial registration: UMIN-CTR (http://upload.umin.ac.jp/cgi-open-bin/ctr/ctr.cgi?function=search&action=input&language=E) UMIN000001102

Citation: Tanuma J, Sano K, Teruya K, Watanabe K, Aoki T, et al. (2013) Pharmacokinetics of Rifabutin in Japanese HIV-Infected Patients with or without Antiretroviral Therapy. PLoS ONE 8(8): e70611. doi:10.1371/journal.pone.0070611

Editor: Omar Sued, Fundacion Huesped, Argentina

Received February 26, 2013; **Accepted** June 19, 2013; **Published** August 5, 2013

Funding: This work was supported by the Health Labour Sciences Research Grant (#H18-AIDS-008 and #H21-AIDS-006) from the Ministry of Health, Labour and Welfare of Japan. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: jtanuma@acc.ncgm.go.jp

Introduction

Rifabutin is commonly used for human immunodeficiency virus (HIV)-associated mycobacterial infections, especially during combination antiretroviral therapy (cART) containing HIV protease inhibitors (PIs), since it is less likely to induce hepatic microsomal enzymes than rifampicin [1–4]. Conversely, rifabutin and its active metabolite, 25–*O*-desacetyl rifabutin, are substrates of CYP 3A4 and concomitant use of PIs can elevate blood concentrations of rifabutin and 25–*O*-desacetyl rifabutin [3–8]. Such rise can increase the risk of side effects such as anterior uveitis [2,9–12]. Thus, a lower dose of rifabutin has been recommended in patients treated with PIs.

The previously recommended dose of rifabutin in combination with ritonavir-boosted PI (PI/r) [13] of 150 mg every other day, was associated with low rifabutin plasma concentrations and increases rate of acquired rifamycin resistance [14–17]. Furthermore, the Tuberculosis Trials Consortium (TBTC)/US Public Health Service Study 23 [14] suggested that AUC0–24 of 4.5 μg/mL is the cutoff value for risk of emergence of resistance to rifamycin. On the other hand, the combination of rifabutin at 150 mg thrice weekly with atazanavir-ritonavir provides exposure to rifabutin comparable to that of rifabutin 300 mg alone [11]. Thus, although 150 mg/day is the current recommended dose for rifabutin during PI/r-based cART [4], the optimal dose of rifabutin when used with a PI/r regimen remains to be established.

Ethnic differences, including body weight, renal clearance and various genetic factors like single nucleotide polymorphism (SNP), haplotype or DNA methylation [18,19], may alter the dose required to achieve a particular concentration of the drug in the circulation. Thus, pharmacokinetic studies involving different ethnic groups are needed to determine the recommended dose that take such factors into account. To our knowledge, there are no such pharmacokinetic studies for rifabutin use in Asians, who

are characterized by lower body weight compared with other ethnic groups. The present study was conducted to evaluate the pharmacokinetics of rifabutin in Japanese patients with HIV-1-related mycobacterial infection when used alone at 300 mg/day without cART and at 150 mg every other day when used in combination with lopinavir/ritonavir.

Methods

Ethics Statement

The study protocol was approved by the Ethics Committee of the National Center for Global Health and Medicine (NCGM-H20–580: approved on 7th February 2008). All participants provided their written informed consent before enrollment as indicated in the protocol.

The protocol for this study and supporting CONSORT checklist are available as supporting information; see File S1 for English translation of the protocol and File S2 for the Japanese original protocol and File S3 for CONSORT checklist.

Study design

Consecutive patients with HIV-1-related mycobacterial infection who received rifabutin-containing therapy at the National Center for Global Health and Medicine, Tokyo, Japan, between February 2008 and March 2009, were eligible for the study. After their written informed consent was provided, clinical history, physical examinations and laboratory tests (e.g., blood chemistry and complete blood cell count) were carried out within one week prior to the pharmacokinetic study. Patients were excluded if they were over 20 years of age or if they had abnormal liver function tests [aspartate aminotransferase (AST), alanine aminotransferase (ALT) or total bilirubin (>3 times the upper limit of normal: ULN)], or severe renal dysfunction (creatinine clearance <30 ml/min), and in the case of female patients if they were pregnant or breastfeeding. Rifabutin was administered while fasting at 300 mg/day and the dose was adjusted when used with cART as recommended by the treatment guideline at the time of the study [13]. Medications administered concomitantly or within 2 weeks before the first study day were recorded. To evaluate the impact of rifabutin plasma concentration on treatment efficacy and adverse events, participants were followed up for at least 2 years after stopping rifabutin. Any side effect noted during rifabutin use or within four weeks after stopping rifabutin, its association with rifabutin was assessed.

Pharmacokinetic assays

Pharmacokinetic sampling commenced after 5 days of rifabutin-containing anti-mycobacterial therapy without (Group I) or with (Group II) cART. Sequential enrollment of a patient into both groups was accepted. Blood samples were collected just before rifabutin administration and then 0.5, 1, 2, 4, 6, 8 and 24 hours afterward. Patients of Group II treated with 150 mg of rifabutin every other day underwent additional sampling at 48 hours. The plasma concentrations of rifabutin and its major metabolite, 25-O-desacetyl rifabutin [20–23] were determined simultaneously by validated high-pressure liquid chromatography (HPLC). Blood samples were taken in heparin-containing tubes, placed on ice and centrifuged at 3000×g for 10 min. Then, the obtained plasma was deproteinized by using three times volume of methanol and centrifuged 15,000×g for 5 min, and the supernatant was used for assay. The HPLC standard for rifabutin and 25-O-desacetyl rifabutin were kindly provided by Pfizer Co. (Pfizer, Inc., NY). The HPLC system consisted of Agilent 1100 series (Agilent Technologies, Santa Clara, CA). Isocratic elution was performed using the Inertsil ODS-3 column (5 μm, 4.6 mm I.D. ×150 mm; GL Sciences Inc, Tokyo, Japan) with a guard column (5 μm, 4.6 mm I.D. ×10 mm; GL Sciences Inc). The UV detection wavelength was 280 nm. The mobile phase consisted of 9 mM

Table 1. Characteristics of study subjects.

	All (n = 16)	Group I (without cART, n = 9)	Group II (with cART, n = 7)	*p* value [a]
Male sex, n	16	9	7	
Age, median years (range)	36 (23–60)	36 (23–55)	35 (23–60)	0.53
Body weight, median kg (range)	57.3 (44–66)	58.0 (46–64)	56.5 (44–66)	0.98
Mycobacterium, multiple choice, n				
M. tuberculosis	13	7	6	1.00
M. avium	4	3	1	0.94
M. kansasii	1	0	1	0.85
CD4 count, median cells/mm^3 (range)	63 (2–164)	63 (2–164)	63 (19–135)	0.84
Plasma viral load, median log copies/ml (range)	4.97 (3.43–6.62)	4.98 (4.18–6.62)	4.95 (3.43–5.18)	0.10
AST, median IU/L (range)	29 (16–70)	25 (16–59)	30 (17–51)	0.65
ALT, median IU/L (range)	27 (13–70)	26 (23–70)	29 (19–70)	0.31
Time on rifabutin, median days (range)	15 (5–63)	7 (5–20)	29 (10–63)	0.017
Time on cART, median days (range)	14 (10–29)	–	14 (10–29)	–
Concomitant medications, n				
lopinavir-ritonavir	7	–	7	–
clarithromycin	3	2	1	1.00
fluconazole	1	0	1	0.85

[a]By Fisher's exact test for categorical data and Mann Whitney's U test for continuous variables.
cART, combination antiretroviral therapy; AST, aspartate aminotransferase; ALT, alanine aminotransferase; IU, international unit.
doi:10.1371/journal.pone.0070611.t001

phosphate buffer (pH 6.8)-acetonitrile (30:70, v/v). The flow-rate was set at 1.0 ml/min and all separations were performed at 30°C in column oven.

Statistical and pharmacokinetic analyses

The area under the curve (AUC) was calculated using non-compartmental techniques (WinNonlin, ver. 5, Pharsight Corp., Mountain View, CA) based on the obtained values (AUC 0–24 h for all, AUC 0–48 h for Group II). The maximum plasma concentration (C_{max}) and time of C_{max} (T_{max}) were determined directly from the data.

Statistical analyses were performed using SPSS software package for Windows, version 17.0J (SPSS Japan Inc, Tokyo). Differences between groups were determined by using the Fisher's exact test for categorical data and the Mann Whitney's test for continuous variables. For all statistical analyses, differences were considered significant if the p value was less than 0.05.

Results

Patient characteristics

A total of 15 patients were enrolled in the study and 5 of 15 participated in both Group I and II. In total, twenty sampling was done for rifabutin pharmacokinetic analysis; 11 in Group I and 9 in Group II. Data from two sampling in Group I and 2 in Group II were excluded from the analysis because samples at 24-hour were unavailable or sampling was conducted earlier than 5 days of rifabutin use. As a result, data from 9 sampling in Group I and 7 sampling in Group II were used for analysis. The baseline characteristics of the 16 sampling cases are summarized in Table 1. All 7 patients of Group II were being treated with lopinavir/ritonavir as their cART, and thus rifabutin was administered at 150 mg every other day based on the guidelines at the time of the study [13]. Two cases of Group I and 1 of Group II were being treated with clarithromycin (CAM) [20] for systemic mycobacterial infection caused by *M. avium* or *M. intracullulare* (*M. avium* Complex: MAC). Five patients of Group I, in whom ART had been delayed several weeks after anti-mycobacterial therapy to prevent the immune reconstitution inflammatory syndrome (IRIS), were later enrolled in the study as patients of Group II (Figure 1). Accordingly, the median time of rifabutin use was longer in Group II than in Group I. There was no significant difference between the groups with regard to gender, age, body weight, CD4 counts, HIV-RNA load, type of mycobacteria and concomitant use of clarithromycin or fluconazole. All were Japanese and the median body weight was 57.3 kg. All patients completed their anti-

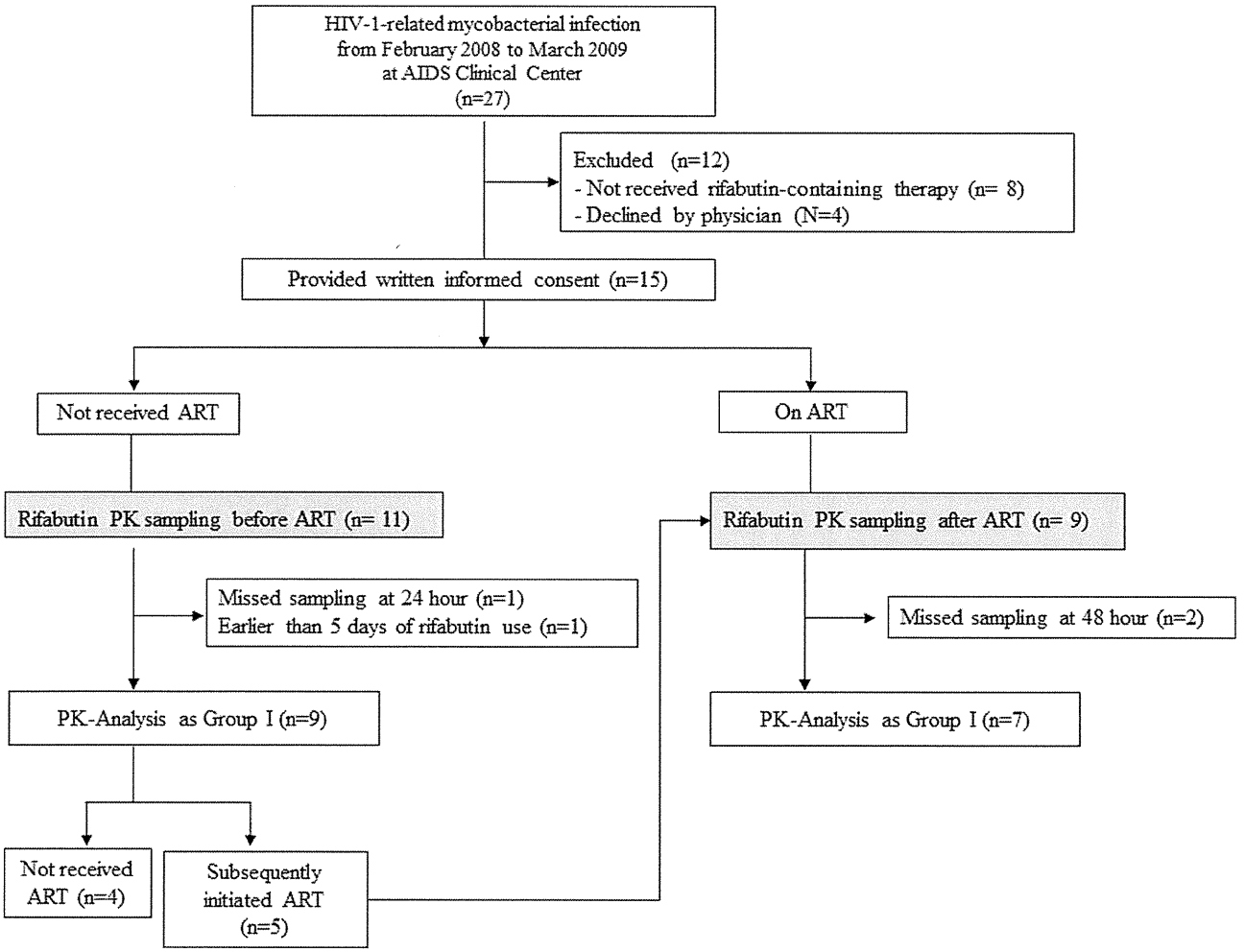


Figure 1. Flow chart of participants through the study. PK, pharmacokinetic; ART, antiretroviral therapy.
doi:10.1371/journal.pone.0070611.g001

Table 2. Pharmacokinetic parameters for rifabutin and 25-*O*-desacetyl rifabutin.

	Group I (without combination antiretroviral therapy, n = 9)				Group II (with combination antiretroviral therapy, n = 7)				*P* value[a]
	Median (range)		Mean (90% CI)		Median (range)		Mean (90% CI)		
Rifabutin									
C_{max} (μg/mL)	0.46	(0.15–0.86)	0.44	(0.39–0.49)	0.28	(0.10–0.44)	0.29	(0.25–0.33)	0.10
AUC_{0-24} (μg h/mL)	2.79	(1.32–15.7)	4.86	(3.83–5.90)	3.00	(1.13–5.43)	3.38	(2.92–3.84)	0.38
AUC_{0-48} (μg h/mL)[b]	5.59	(2.63–31.3)	9.71	(7.62–511.8)	4.21	(1.76–6.90)	4.58	(3.38–5.78)	0.32
T_{max} (h)	2.0	(2.0–4.0)	2.9	(2.6–3.1)	6.0	(2.0–12.0)	4.8	(4.1–5.1)	0.03
25-*O*-desacetyl rifabutin									
C_{max} (μg/mL)	0.00	(0.00–0.30)	0.05	(0.03–0.08)	0.13	(0.05–0.23)	0.14	(0.12–0.16)	0.05
AUC_{0-24} (μg h/mL)	0.00	(0.00–3.69)	0.82	(0.45–1.20)	1.52	(0.44–3.64)	2.07	(1.62–2.52)	0.12
AUC_{0-48} (μg h/mL)[b]	0.00	(0.00–7.38)	1.64	(0.89–2.39)	5.93	(0.44–7.21)	4.32	(3.27–5.38)	0.15
T_{max} (h)	6.0	(2.0–8.0)	5.3	(4.6–6.0)	6.0	(2.0–12.0)	5.7	(4.6–6.9)	0.87
Rifabutin plus 25-*O*-desacetyl rifabutin									
C_{max} (μg/mL)	0.47	(0.15–0.99)	0.49	(0.40–0.52)	0.42	(0.16–0.56)	0.39	(0.34–0.44)	0.54
AUC_{0-24} (μg h/mL)	3.36	(1.32–19.3)	5.49	(4.18–6.76)	6.23	(1.57–7.92)	5.27	(4.48–6.07)	0.93
AUC_{0-48} (μg h/mL)[b]	6.72	(2.63–38.7)	10.9	(8.35–13.5)	6.80	(2.20–14.1)	7.95	(6.40–9.49)	0.46

[a]By the Mann Whitney's *U* test.
[b]In Group I, AUC_{24-48} is assumed the same as AUC_{0-24} and AUC_{0-48} is calculated as double of AUC_{0-24} for comparison with Group II.
C_{max}, maximum plasma concentration; AUC, area under the curve; T_{max}, time of C_{max}; CI, confidence interval.
doi:10.1371/journal.pone.0070611.t002

mycobacterial treatment with clinical resolution of mycobacterial infections. None of the participants had treatment failure or relapse within more than 3 years of observation. Worsening of intra-abdominal lymphadenitis was observed in one patient with systemic M. avium infection at 8 months after stopping the 2-year rifabutin-containing anti-mycobacterial therapy, which excluded treatment failure or relapse. All patients confirmed complete adherence to anti-mycobacterial therapy and cART.

Pharmacokinetic parameters of rifabutin and its 25-*O*-desacetyl metabolite

The pharmacokinetic parameters of rifabutin and 25-*O*-desacetyl rifabutin are summarized in Table 2 and their mean plasma concentration-time data of 48 hours are illustrated in Figure 2A and 2B. For calculation of AUC_{0-48}, the data from 24 to 48 hours in Group I was assumed to be the same as that for 0–24 hours because rifabutin was administered once a day at the same dosage. As shown in Table 2, the mean values of C_{max} and AUC_{0-24} of rifabutin were 36% and 26% lower in Group II than in Group I, while the mean values of C_{max} and AUC_{0-24} of 25-*O*-desacetyl rifabutin were 186% and 152% higher in Group II than in Group I. However, the differences in the above values between the two groups were not statistically different. The low rifabutin concentration and high metabolite concentration in Group II may reflect the induction of rifabutin metabolism due to the longer duration of rifabutin use. Since 25-*O*-desacetyl rifabutin is microbiologically active against mycobacterium, total rifabutin activity might include rifabutin plus this metabolite. Figure 2C illustrates the mean plasma concentration of rifabutin plus the metabolite over time. Patients of Groups I and II had similar plasma concentrations of rifabutin plus the metabolite within the first 24 hours. However, the level of rifabutin plus the metabolite during the subsequent 24–48 hours was considerably lower in Group II than in Group I (dotted line in Figure 2C: Group I during 0–24 hours), whereas the AUC_{0-48} was not statistically different between the groups. Notably, 6 (67%) cases of Group I and 5 (71%) of Group II failed to achieve the AUC_{0-24} value suggested as risk for emergence of rifamycin-resistant *M. tuberculosis* [14] (4.5 μgh/mL). Neither C_{max} nor AUC_{0-24} of rifabutin and 25-*O*-desacetyl rifabutin were associated with age, body weight, body mass index, or CD4 count.

Rifabutin-associated side effects

Of the 15 participants, three patients developed side effects possibly related to rifabutin during the observational period; two of Group I developed skin rash and the other of Group II developed grade 2 rise in liver enzymes (ALT or AST 2.6–5.0 times of ULN). The skin rash appeared on day 11 of rifabutin-containing regimen in one patient and on day 28 in the other, and was resolved in both patients within several days after withdrawal of rifabutin. The rise in liver enzymes was detected after two months of rifabutin-containing regimen in combination with cART, and improved soon after discontinuation of rifabutin. Notably, the median CD4 counts in the three patients with rifabutin toxicity were significantly lower than in patients without rifabutin toxicity (12 *vs* 76, cells/mm^3, $p = 0.028$). However, rifabutin toxicity did not correlate with rifabutin AUC_{0-24}, C_{max}, or the concurrent use of cART (rifabutin AUC_{0-24}: $p = 0.37$, rifabutin C_{max}: $p = 0.86$, cART use: $p = 0.21$).

Discussion

In the present study, a low dose of rifabutin (150 mg every other day), in combination with lopinavir/ritonavir-containing cART, yielded comparable AUC_{0-24} of rifabutin and 25-*O*-desacetyl rifabutin to the commonly used dose of rifabutin of 300 mg/day. The advantage of the low-dose rifabutin included lower exposure to rifabutin and metabolite during the subsequent 24 to 48 hours in Japanese patients with HIV-mycobacteria co-infection. Since many participants started their cART after at least 1 month of

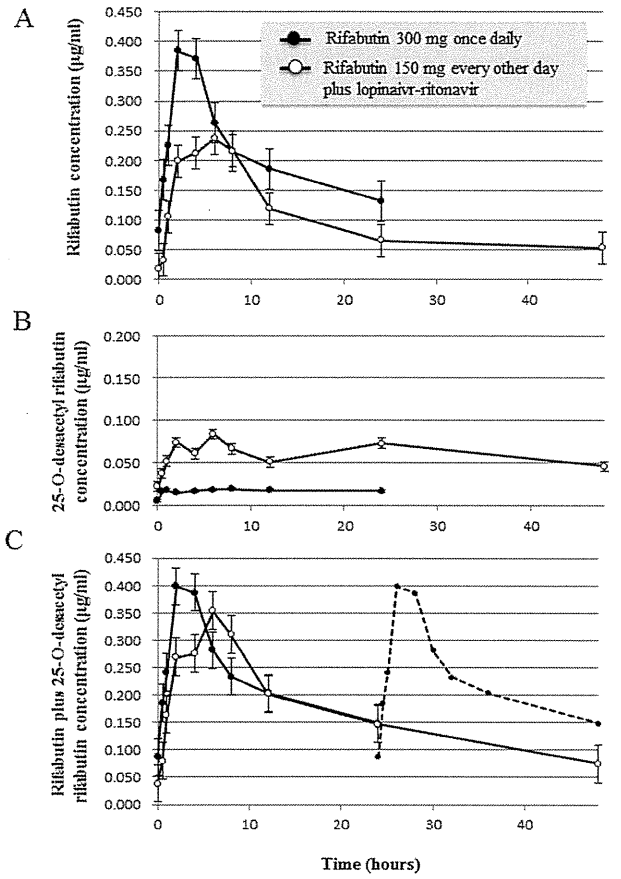


Figure 2. Mean plasma concentrations-versus-time plots of rifabutin (A), 25-*O*-desacetyl rifabutin (B), and rifabutin plus 25-*O*-desacetylrifabutin (C). Nine patients of Group I received 300 mg of rifabutin and 7 patients of Group II received 150 mg of rifabutin every other day with lopinavir/ritonavir-containing antiretroviral therapy. *Solid circles:* Group I, *open circles:* Group II. Data are mean ±1 standard errors. Dotted line in Figure C represents data of Group I during 0–24 hour for reference. RBT, rifabutin; PI/r, ritonavir-boosted protease inhibitor.
doi:10.1371/journal.pone.0070611.g002

anti-mycobacterial therapy in order to avoid deterioration by immune-reconstitution syndrome, the metabolism of rifabutin was induced upon the commencement of cART. This led to lower rifabutin concentration and higher 25-*O*-desacetyl rifabutin concentration in Group II but provided similar concentrations of rifabutin plus its active metabolite. However, on the day without medication, plasma concentrations of rifabutin and its active metabolite were lower in Group II, which were less than the susceptibility breakpoint level for *M. tuberculosis* proposed by others [20]. This suggests increased risk of emergence of rifamycin-resistant *M. tuberculosis* during the day without medication under low-dose rifabutin therapy, and that the currently recommended dosage 150 mg daily with PI/r is reasonable to this population as well. In this regard, Zhang et al. [11] reported that treatment with 150 mg/day rifabutin with atazanavir-ritonavir resulted in high risk of severe neutropenia. Furthermore, their post-hoc simulation showed that rifabutin 150 mg thrice weekly with atazanavir-ritonavir provided a comparable exposure to rifabutin compared with rifabutin 300 mg daily. Considering the risk of rifamycin-resistance and rifabutin toxicity, monitoring of rifabutin plasma concentration should be considered until the optimal rifabutin dosing during PI/r-based cART is fully established.

Although none of the patients showed treatment failure or relapse in this study, the rifabutin AUC0-24 observed in the study was in general close to the low end of the value reported in previous studies [7,14] and many participants [6 (67%) of Group I and 5 (71%) of Group II] failed to achieve AUC0-24 4.5 μgh/mL, the cutoff value suggested as risk for emergence of rifamycin-resistant M. tuberculosis [14]. One of the reasons for this discordant result might be the limitation of our study of small sample size involving several MAC and M. kansasii infections. Since acquisition of rifamycin-resistance M. tuberculosis was not frequent enough in this study group, it was difficult to evaluate the association with rifabutin pharmacokinetics and emergence of rifamycin-resistance. Other reasons may be the biological characteristics of rifabutin. Rifabutin has long postantibiotic effect against M. tuberculosis and MAC [20], shows extensive distribution in various tissues [21,22], and readily penetrates cell membranes of leucocytes [21,22]. These characteristics and their variations among patients can considerably influence the outcome of rifabutin-containing anti-mycobacterial therapy and therefore might be one of the explanations of favorable efficacy despite lower plasma concentrations of rifabutin in our study. Another limitation of this study is that plasma concentration of isoniazid was not measured, although low isoniazid plasma concentration is known to be independently related to treatment failure of HIV/TB co-infection [24]. Additionally, although there was no difference in rifabutin concentration among the patients with or without use of clarithromycin or fluconazole, those drugs can increase the rifabutin AUC and possibly affect the results. Since our study was enrolling patients with heterogeneous backgrounds in the real clinical setting, such as timing of sampling or different combination of anti-mycobacteial drugs, it was difficult to completely eliminate those impacts from the analysis. These conditions should be taken into account in the assessment of treatment outcome and associated factors in this study.

Among 15 study participants, 3 patients developed side effects related to rifabutin therapy, including skin rash and rise in liver enzymes. Notably, their CD4 counts were lower than those who did not show rifabutin toxicity, although rifabutin plasma concentrations and the concurrent use of cART were similar in the two groups. This is the first report implicating low CD4 count as a risk factor for rifabutin-related side effects. However, like other side effects of rifabutin, such as uveitis and leukocytopenia, which have been reported to be related to high-dose rifabutin or high rifabutin plasma concentrations [9–12], careful assessment involving larger population samples are needed to evaluate the association between high plasma concentrations of rifabutin and the related skin rash and hepatotoxicity.

In conclusion, in Japanese patients with HIV-mycobacteria co-infection, the plasma concentrations of rifabutin and active metabolite within the first 24 hours of treatment with low-dose rifabutin (150 mg every other day) combined with lopinavir-ritonavir, were similar to those encountered with 300 mg/day rifabutin alone. However, these concentrations decreased on the day without medication. Our findings could help determine the optimal dose of rifabutin during cART. Further studies are needed to establish the optimal dose of rifabutin during cART. Monitoring of rifabutin plasma concentration should be considered in patients with HIV-mycobacteria co-infection.

Supporting Information

Protocol S1 Summary in English. English translation of the protocol Summary.
(DOCX)

Protocol S1 Protocol and IC form in Japanese. The full version of the study protocol and the informed consent form in Japanese.
(PDF)

CONSORT Checklist S2.
(DOC)

References

1. American Thoracic Society, Centers for Disease Control and Prevention and the Infectious Diseases Society. (2003) AmericanThoracicSociety/Centers for Disease Control and Prevention/Infectious Diseases Society of America: treatment of tuberculosis. Am J Respir Crit Care Med 6: 603–662.
2. Pfizer, Inc. Mycobutin (rifabutin) prescribing information. Pfizer, Inc., New York, NY. Available: http://www.pfizer.com/files/products/uspi_mycobutin.pdf. Accessed 2012 August 21.
3. Centers for Disease Control and Prevention. (2009) Guidelines for Prevention and Treatment of Opportunistic Infections in HIV-Infected Adults and Adolescents. MMWR 58: 1–207.
4. Panel on Antiretroviral Guidelines for Adults and Adolescents. Guidelines for the use of antiretroviral agents in HIV-1-infected adults and adolescents.Department of Health and Human Services. Available: http://aidsinfo.nih.gov/contentfiles/lvguidelines/AdultandAdolescentGL.pdf. Accessed 2012 August 21.
5. Kraft WK, McCrea JB, Winchell GA, Carides A, Lowry R, et al. (2004) Indinavir and rifabutin drug interactions in healthy volunteers. J Clin Pharmacol 44: 305–313.
6. Ford SL, Chen YC, Lou Y, Borland J, Min SS, et al. (2008) Pharmacokinetic interaction between fosamprenavir-ritonavir and rifabutin in healthy subjects. Antimicrob Agents Chemother 52: 534–538.
7. Boulanger C, Hollender E, Farrell K, Stambaugh JJ, Maasen D, et al. (2009) Pharmacokinetic evaluation of rifabutin in combination with lopinavir-ritonavir in patients with HIV infection and active tuberculosis. Clin Infect Dis 49: 1305–1311.
8. Zhang X, Fettner S, Zwanziger E, Rowell L, Salgo M, et al. (2011) Pharmacokinetic interaction study of ritonavir-boosted saquinavir in combination with rifabutin in healthy subjects. Antimicrob Agents Chemother 55: 680–687.
9. Centers for Disease Control and Prevention. (1994) Uveitis associated with rifabutin therapy. MMWR 43: 658.
10. Shafran SD, Singer J, Zarowny DP, Deschênes J, Phillips P, et al. (1998) Determinants of rifabutin-associated uveitis in patients treated with rifabutin, clarithromycin, and ethambutol for *Mycobacterium avium* complex bacteremia: a multivariate analysis. Canadian HIV Trials Network Protocol 010 Study Group. J Infect Dis 177: 252–255.
11. Zhang J, Zhu L, Stonier M, Coumbis J, Xu X, et al. (2011) Determination of rifabutin dosing regimen when administered in combination with ritonavir-boosted atazanavir. J Antimicrob Chemother 66: 2075–2082.
12. Sekar V, Lavreys L, Van de Casteele T, Berckmans C, Spinosa-Guzman S, et al. (2010) Pharmacokinetics of darunavir/ritonavir and rifabutin coadministered in HIV-negative healthy volunteers. Antimicrob Agents Chemother 54: 4440–4445.
13. Panel on Antiretroviral Guidelines for Adults and Adolescents. (2008) Guidelines for the use of antiretroviral agents in HIV-1-infected adults and adolescents. Department of Health and Human Services. November 3: 1–139. Available: http://www.aidsinfo.nih.gov/ContentFiles/AdultandAdolescentGL.pdf. Accessed 2012 August 21.
14. Weiner M, Benator D, Burman W, Peloquin CA, Khan A, et al. (2005) Association between acquired rifamycin resistance and the pharmacokinetics of rifabutin and isoniazid among patients with HIV and tuberculosis. Clin Infect Dis 40: 1481–1491.
15. Jenny-Avital ER, Joseph K (2009) Rifamycin-resistant Mycobacterium tuberculosis in the highly active antiretroviral therapy era: a report of 3 relapses with acquired rifampin resistance following alternate-day rifabutin and boosted protease inhibitor therapy. Clin Infect Dis 48: 1471–1474.
16. Li J, Munsiff SS, Driver CR, Sackoff J (2005) Relapse and acquired rifampin resistance in HIV-infected patients with tuberculosis treated with rifampin- or rifabutin-based regimens in New York City. 1997–2000. Clin Infect Dis 41: 83–91.
17. Khachi H, O'Connell R, Ladenheim D, Orkin C. et al. (2009) Pharmacokinetic interactions between rifabutin and lopinavir/ritonavir in HIV-infected patients with mycobacterial co-infection. J Antimicrob Chemother 64: 871–873.
18. Gomez A, Ingelman-Sundberg M (2009) Pharmacoepigenetics: its role in interindividual differences in drug response. Clin Pharmacol Ther 85: 426–430.
19. Lee IS, Kim D (2011) Polymorphic metabolism by functional alterations of human cytochrome P450 enzymes. Arch Pharm Res 34: 1799–1816.
20. Kunin CM (1996) Antimicrobial activity of rifabutin. Clin Infect Dis 22: S3–13.
21. Skinner MH, Hsieh M, Torseth J, Pauloin D, Bhatia G, et al. (1989) Pharmacokinetics of rifabutin. Antimicrob Agents Chemother 33: 1237–1241.
22. Blaschke TF, Skinner MH (1996) The clinical pharmacokinetics of rifabutin. Clin Infect Dis 22: S15–21.
23. Strolin Benedetti M, Efthymiopoulos C, Sassella D, Moro E, Repetto M (1990) Autoinduction of rifabutin metabolism in man. Xenobiotica 20: 1113–1119.
24. Weiner M, Burman W, Vernon A, Benator D, Peloquin CA, et al. (2003) Low isoniazid concentrations and outcome of tuberculosis treatment with once-weekly isoniazid and rifapentine. Tuberculosis Trials Consortium. Am J Respir Crit Care Med 167: 1341–1347.

Acknowledgments

We thank all the staff of the AIDS Clinical Center for the care of patients.

Author Contributions

Conceived and designed the experiments: JT. Performed the experiments: JT KS. Analyzed the data: JT. Contributed reagents/materials/analysis tools: JT KS. Wrote the paper: JT. Technical advice: YK. Patients' recruitment: KW TA HH HY K. Tsukada. Technical advice: K. Teruya HG SO.

OPEN ACCESS Freely available online

PLOS | ONE

Preemptive Therapy Prevents Cytomegalovirus End-Organ Disease in Treatment-Naïve Patients with Advanced HIV-1 Infection in the HAART Era

Daisuke Mizushima[1,2], Takeshi Nishijima[1,2], Hiroyuki Gatanaga[1,2]*, Kunihisa Tsukada[1], Katsuji Teruya[1], Yoshimi Kikuchi[1], Shinichi Oka[1,2]

1 AIDS Clinical Center, National Center for Global Health and Medicine, Tokyo, Japan, **2** Center for AIDS Research, Kumamoto University, Kumamoto, Japan

Abstract

Background: The efficacy of preemptive therapy against cytomegalovirus (CMV) infection remains unknown in treatment-naïve patients with advanced HIV-1 infection in the HAART era.

Methods: The subjects of this single-center observation study were126 treatment-naïve HIV-1 infected patients with positive CMV viremia between January 1, 2000 and December 31, 2006. Inclusion criteria were age more than 17 years, CD4 count less than 100/μl, plasma CMV DNA positive, never having received antiretroviral therapy (ART) and no CMV end-organ disease (EOD) at first visit. The incidence of CMV-EOD was compared in patients with and without preemptive therapy against CMV-EOD. The effects of the CMV preemptive therapy were estimated in uni- and multivariate Cox hazards models.

Results: CMV-EOD was diagnosed in 30 of the 96 patients of the non-preemptive therapy group (31%, 230.3 per 1000 person-years), compared with 3 of the 30 patients of the preemptive therapy group (10%, 60.9 per 1000 person-years). Univariate (HR = 0.286; 95%CI, 0.087–0.939; p = 0.039) and multivariate (adjusted HR = 0.170; 95%CI, 0.049–0.602; p = 0.005) analyses confirmed that CMV-EOD is significantly prevented by CMV preemptive therapy. Multivariate analysis showed that plasma CMV DNA level correlated significantly with CMV-EOD (per log10/ml, adjusted HR = 1.941; 95%CI, 1.266–2.975; p = 0.002). Among the 30 patients on preemptive therapy, 7 (23.3%) developed grade 3–4 leukopenia. The mortality rate was not significantly different between the two groups (p = 0.193, Log-rank test).

Conclusions: The results indicate that preemptive therapy lowers the incidence of CMV-EOD by almost 25%. Preemptive therapy for treatment-naïve patients with CMV viremia is effective, although monitoring of potential treatment-related side effects is required.

Citation: Mizushima D, Nishijima T, Gatanaga H, Tsukada K, Teruya K, et al. (2013) Preemptive Therapy Prevents Cytomegalovirus End-Organ Disease in Treatment-Naïve Patients with Advanced HIV-1 Infection in the HAART Era. PLoS ONE 8(5): e65348. doi:10.1371/journal.pone.0065348

Editor: Michael Nevels, University of Regensburg, Germany

Received January 7, 2013; **Accepted** April 24, 2013; **Published** May 28, 2013

Funding: This work was supported by a Grant for National Center for Global Health and Medicine (23-114). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: higatana@acc.ncgm.go.jp

Introduction

Although the incidence of new cases of cytomegalovirus (CMV) end-organ disease (EOD) has decreased by 75%–80% with the advent of antiretroviral therapy (ART) and is currently estimated to be <6 cases per 100 person-years [1], CMV-EOD is still one of the major debilitating diseases among patients with advanced HIV infection.

CMV preemptive therapy is commonly used for patients scheduled for hematopoietic cell transplantation and solid organ transplantation, with clinical evidence of efficacy[2–6], however, it is not generally recommended in HIV patients [7] because of concerns regarding cost-effectiveness, risk of developing CMV resistance, side effect and the lack of a proven survival benefit [8]. A prospective trial in cooperation with Roche company to evaluate the efficacy of preemptive therapy in the pre-HAART (highly active ART) era showed significant preventive effect of oral ganciclovir (GCV) [9]. However; other studies conducted in both pre-HAART and HAART eras showed no significant effect [10,11]. However, the above studies included patients who had previously received ART. Therefore, the efficacy of preemptive therapy against CMV infection remains unknown in treatment-naïve patients with advanced HIV-1 infection in the HAART era.

We retrospectively compared the incidence of CMV-EOD in a cohort of ART-naïve adult patients with advanced HIV infection (low CD4 count and plasma CMV-DNA-positive). One group of these patients had received CMV preemptive therapy, while the other had not received such therapy.

Methods

Ethics Statement

The study was approved by the Human Research Ethics Committee of National Center for Global Health and Medicine,

Tokyo. All patients included in this study provided a written informed consent for their clinical and laboratory data to be used and published for research purposes. This study has been conducted according to the principles expressed in the Declaration of Helsinki.

Study design

We performed a retrospective, single-center cohort study to elucidate the effectiveness of preemptive CMV treatment in HIV-infected patients with positive CMV viral load in the prevention of CMV-EOD. The study was conducted at the National Center for Global Health and Medicine, Tokyo, one of the largest clinics for patients with HIV infection in Japan, with more than 2,700 registered patients as of December 2006. The study population comprised treatment-naïve HIV infected patients aged more than 17 years, with CD4 count less than 100/μl and positive plasma CMV DNA viral load, who presented for the first time at our hospital between January 1, 2000 and December 31, 2006. Those with CMV-EOD at presentation and those with <3 months of follow-up were excluded. The follow-up period was 2 years from the initial visit.

Definition of CMV-EOD and CMV preemptive therapy

CMV-EOD was diagnosed according to standardized ACTG criteria (see Table S1) [11]. CMV retinitis was routinely screened for by dilated indirect ophthalmoscopy at both the first visit to the hospital and a few months after the commencement of ART. Other evaluations, such as endoscopy and bronchoscopy, were carried out in response to the symptoms and clinical condition. The diagnosis of CMV-EOD was established by at least two experts from our hospital.

CMV preemptive therapy was prescribed based on the clinician's assessment. CMV preemptive therapy was provided at our institution for patients with plasma CMV DNA of >5000 copies/ml. For patients with plasma CMV DNA of >3000 but less than 5000 copies/ml, the decision to initiate preemptive therapy was left to the attending physician, taking into consideration the overall clinical condition, such as subsequent rise in plasma CMV DNA and/or use of immunosuppressants, such as steroids and chemotherapeutic agents. Ganciclovir (GCV) and valganciclovir (VGCV) were the most commonly used agents, followed by foscarnet (FOS). The choice of induction (intravenous GCV 5 mg/kg every 12 hours, oral VGCV900 mg twice a day or intravenous FOS 90 mg/kg every 12 hours) or maintenance dose (intravenous GCV 5 mg/kg every 24 hours, oral VGCV 900 mg a day or intravenous FOS 90 mg/kg every 24 hours) was based on the clinical condition, such as the level of plasma CMV DNA or state of immunosuppression. The duration of therapy varied across individuals. CMV preemptive therapy was defined as at least a 7-day treatment with agents effective against CMV. The normal course of CMV preemptive therapy was 2 weeks of GCV induction dose followed by VGCV or GCV maintenance dose until plasma CMV DNA became negative. Patients were retreated based on clinicians' decision under some conditions with high risks for CMV-EOD as described above, if plasma CMV DNA became positive again after preemptive therapy.

Measurements

Plasma CMV DNA was measured using real-time PCR with a lower limit of detection of 200 copies/mL(CMV geniQ, Bio Medical Laboratory, Inc., Tokyo, Japan). Plasma CMV DNA was measured routinely at the first visit in patients with CD4 count of <100/μl, and re-examined every week or monthly, according to the level of plasma CMV DNA viral load or immune status and at the discretion of the attending physician.

In this study, the primary exposure variable was CMV preemptive therapy over no CMV preemptive therapy. The potential risk factors for CMV-EOD were determined based on previous studies [12–18], and included basic demographics and laboratory data, including age, sex, CD4 cell count, HIV viral load, plasma CMV DNA, and presence or absence of other medical conditions (concurrent use of steroids, concurrent chemotherapy and concurrent AIDS-defining diseases). For each patient, data on or closest to the day of the first visit to our hospital were retrieved for analysis.

Statistical analysis

Categorical and continuous baseline demographics and laboratory data were analyzed using Pearson's chi-square test and Student's t-test, respectively. The time from the first visit to our hospital to the development of CMV-EOD was analyzed by the Kaplan Meier method for patients on CMV preemptive therapy and no CMV preemptive therapy, and the log-rank test was used to determine the statistical significance. Censored cases represented those who died, dropped out, or were referred to other facilities before the end of follow-up period. The Cox proportional hazards regression analysis was used to estimate the impact of CMV preemptive therapy on the incidence of CMV-EOD. The impact of basic demographics, baseline laboratory data, and other medical conditions was also estimated with univariate Cox proportional hazards regression.

To estimate the unbiased prognostic impact of CMV preemptive therapy, we used three models based on multivariate Cox proportional hazards regression analysis. Model 1 was the aforementioned univariate analysis for CMV preemptive therapy. Model 2 included age and sex, plus Model 1, in order to adjust for basic characteristics. In Model 3, we added variables with significant relation to CMV-EOD by univariate analysis or assumed as risk factor(s) for CMV-EOD in the literature[12–20] (e.g., CD4 count per 1/μl decrement, HIV viral load per log10/ml, CMVDNA viral load per log10/ml, concurrent steroid use, concurrent chemotherapy and concurrent AIDS defining disease). Statistical significance was set at two-sided p values <0.05. We used hazard ratios (HRs) and 95% confidence intervals (95%CIs) to estimate the impact of each variable on CMV-EOD. All statistical analyses were performed with The Statistical Package for Social Sciences ver. 17.0 (SPSS, Chicago, IL).

Results

Of the 199 HIV-infected patients with CD4 count <100/μl and positive plasma CMV DNA viral load referred to our hospital between January 1, 2000 and December 31, 2006, 126 patients were recruited in the study. Of these, 96 patients received CMV preemptive therapy while 30 did not (Figure 1). Table 1 lists the demographics, laboratory data, and medical conditions of the study population at baseline. The majority of the study population were males, East Asians, and relatively young (median: 42 years). There were no differences in baseline CD4 count ($p = 0.595$) and HIV viral load ($p = 0.628$) between the two groups. Patients of the CMV preemptive therapy group had higher plasma CMV DNA viral load ($p<0.001$), more likely to have developed AIDS defining diseases ($p = 0.042$), and tended to have been treated concurrently with steroids ($p = 0.009$), compared with the non-CMV preemptive group. There were no significant differences in the use of chemotherapy ($p = 1.000$) and in time to initiation of ART since study entry ($p = 0.393$, Table 1) between the two groups.

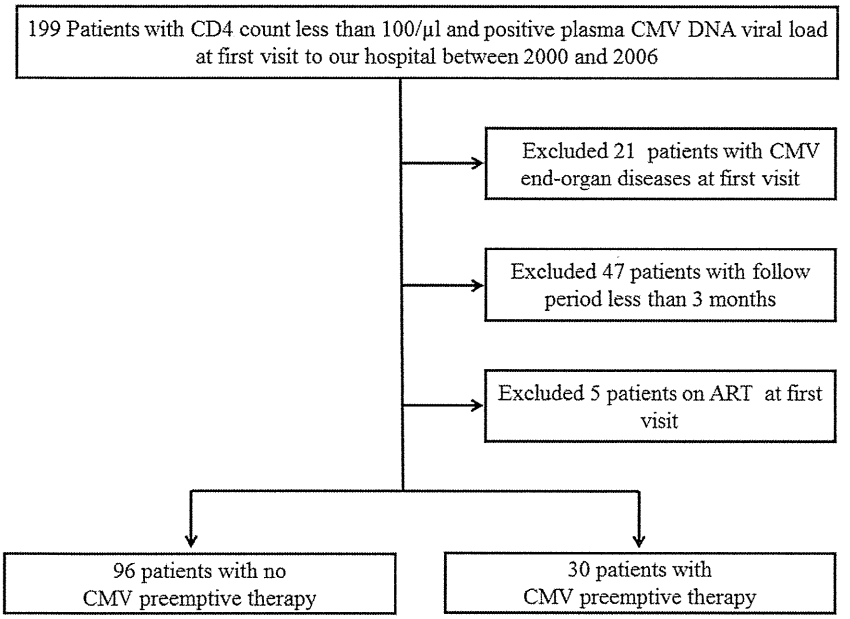


Figure 1. Flow chart of inclusion and exclusion criteria. Of the 199 subjects, 73 were excluded and the remaining 126 were included in the study. The latter group was divided into the preemptive therapy group (n = 30) and the non-therapy group (n = 96).
doi:10.1371/journal.pone.0065348.g001

During the follow-up period, CMV-EOD occurred in 3 (10.0%) patients of the preemptive therapy group and 30 (31.3%) of the non-preemptive therapy group, with an estimated incidence of 60.9 and 230.3 per 1000 person-years, respectively. Figure 2 depicts the time from the first visit to our hospital to the development of CMV-EOD by Kaplan Meier method in the two groups. The incidence of new cases of CMV-EOD was significantly higher in the non-preemptive therapy group, compared with the preemptive therapy group ($p = 0.027$, Log-rank test). The median time from the first visit to the diagnosis of CMV-EOD was 67 days (range, 25–67) for the preemptive therapy group, and 54 days (range, 14–326 days) for the non-preemptive therapy group.

Univariate analysis showed a significant relationship between CMV preemptive therapy and low incidence of CMV-EOD (HR = 0.286; 95%CI, 0.087–0.939; $p = 0.039$) (Table 2). On the other hand, high CMV viral load and HIV viral load tended to be associated with CMV-EOD, while old age, low baseline CD4

Table 1. Baseline demographics and laboratory data of patients who did and did not receive CMV preemptive therapy.

	Non-preemptive therapy (n = 96)	Preemptive therapy (n = 30)	P value
Sex (male), n (%)	88 (91.7)	29 (96.7)	0.685
Median (range) age	41 (24–76)	44 (25–66)	0.729
Ethnicity, n (%)			
East Asians	86 (89.5)	29 (96.7)	
Southeast Asian	5 (5.2)	0 (0.0)	
Black	3 (3.1)	0 (0.0)	
White	2 (2.1)	1 (3.3)	
Median (range) CD4 count (/μl)	28.0 (0–97)	35.5 (3–87)	0.595
Median (range) HIV RNA viral load (log10/ml)	5.3 (3–6)	5.35 (4–7)	0.628
Median (range) CMVDNA viral load (log10/ml)	3.0 (2–5)	4.3 (2–5)	<0.001
Concurrent AIDS, n (%)	78 (81.3)	29 (96.7)	0.042
Steroid use, n (%)	38 (39.6)	20 (66.7)	0.009
Chemotherapy, n (%)	9 (9.4)	2 (6.7)	1.000
Median (range) time (days) to ART*	66 (2–399)	59 (13–158)	0.393
Median (range) follow-up (days)	730 (14–730)	730 (25–730)	0.064

*11 missing values.
Categorical and continuous variables were analyzed using Pearson's chi-square test and Student's t-test, respectively.
doi:10.1371/journal.pone.0065348.t001

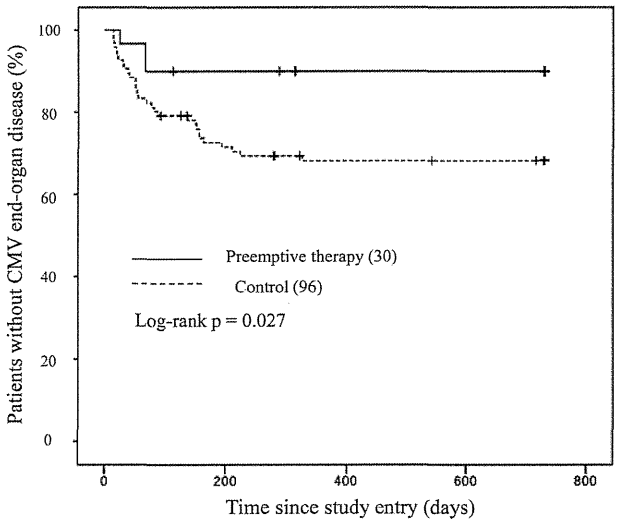


Figure 2. Kaplan-Meier curve showing the time to development of cytomegalovirus (CMV)- end-organ disease (EOD) in the preemptive and non-preemptive therapy groups. Compared to patients on CMV preemptive therapy, those who did not receive preemptive therapy were more likely to develop CMV-EOD (p = 0.027, Log-rank test).
doi:10.1371/journal.pone.0065348.g002

count, use of steroids, chemotherapy, and concurrent AIDS defining diseases were not associated with CMV-EOD. Multivariate analysis identified CMV preemptive therapy as a significant preventive factor against CMV-EOD after adjustment for age and sex (Model 2; adjusted HR = 0.289; 95%CI, 0.088–0.949; p = 0.041, Table 3), and after adjustment for other risk factors (Model 3; adjusted HR = 0.172; 95%CI, 0.049–0.602; p = 0.005, Table 3). In addition, multivariate analysis showed that high CMV viral load correlated significantly with CMV-EOD (Model 3; adjusted HR = 1.941; 95%CI, 1.266–2.975; p = 0.002, Table 3).

Of the 33 patients with CMV-EOD, 22 (66.7%) developed CMV retinitis, 4 (12.1%) developed esophagitis, 3 (9.1%) developed gastroduodenitis, 6 (18.2%) developed colitis and 1 (3.0%) developed pneumonitis. All 3 patients with CMV-EOD of the preemptive therapy group developed retinitis (Table 4).

Table 2. Results of univariate analysis to estimate the risk of various factors in inducing CMV end-organ disease.

	Hazard ratio	95% CI	P value
CMV preemptive therapy	0.286	0.087–0.939	0.039
Female	1.284	0.392–4.209	0.680
Age per 1 year	0.982	0.951–1.013	0.240
CD4 count per 1/μl decrement	1.001	0.989–1.013	0.867
HIV viral load per log10/ml	1.875	0.905–3.884	0.091
CMV viral load per log10/ml	1.450	0.984–2.136	0.060
Use of steroid	0.716	0.356–1.439	0.348
Chemotherapy	1.390	0.488–3.955	0.537
Concurrent AIDS	0.703	0.290–1.704	0.436

CI: confidence interval
The Cox proportional hazards regression analysis was used.
doi:10.1371/journal.pone.0065348.t002

Of 30 patients who received preemptive therapy, 20 (66.7%) received an induction dose of GCV, and 7 patients (23.3%) received maintenance dose. The remaining agents used for preemptive therapy were an induction dose of VGCV, a maintenance dose of FOS and an induction dose of cidofovir. The duration of the preemptive therapy varied between 7 days and 2 months. The following side effects were noted in patients on CMV preemptive therapy: grade 3/4 leukopenia (n = 7, 23.3%) and grade 2 hypercreatininemia (n = 1, 3.3%). Both side effects developed during the use of GCV. Five patients (5.2%) of the non-preemptive therapy group and 4 patients (13.3%) of the preemptive therapy group died during the study period. Of the former group, 3 deaths were due to opportunistic infections (cryptococcus meningitis, non-tuberculous mycobacterial infection and *Pneumocystis jiroveci* pneumonia), 1 due to bacterial infection (sepsis), and 1 due to suicide. Of the latter group, 2 deaths were due to opportunistic infections (malignant lymphoma and *P. jiroveci* pneumonia) and 2 due to bacterial infection (bacterial pneumonias). Deaths and bacterial infections related to preemptive therapy were not observed in our study. The mortality rate was not significantly different between the two groups (p = 0.193, Log-rank test, Figure 3).

Discussion

The results of this observational cohort of treatment-naïve HIV-infected patients with positive plasma CMV DNA showed a significantly lower incidence of CMV-EOD by one-fourths in the CMV preemptive therapy group than in the non-preemptive therapy group, over the 2-year observation period. This finding was significant, despite higher risk for CMV-EOD in the preemptive therapy group, such as higher plasma CMV DNA, higher prevalence of concurrent AIDS defining diseases and more concurrent steroid use, compared with the other group. Univariate and multivariate analyses identified anti-CMV preemptive therapy as a significant preventive factor against CMV-EOD.

Our study is the first to illustrate the significance of anti-CMV preemptive therapy in treatment- naïve HIV-infected patients with CMV viremia and CD4 count less than 100/μl in the HAART era. The hazard ratio of development of CMV-EOD decreased by 82.8% following preemptive therapy, compared with no preemptive therapy, even after adjustment for plasma CMV DNA viral load and other factors. The current guidelines do not generally recommend anti-CMV preemptive therapy although this is based on sparse evidence, such as cost effectiveness, CMV resistance, and drug side effects [7]. However, our study suggests that preemptive therapy is a feasible option, if the effective target of preemptive therapy could be selected. Furthermore, the study confirmed that plasma CMV DNA, a known risk factor for CMV-EOD [12–18], was a significant independent risk factor.

A few prospective clinical trials investigated the efficacy of preemptive therapy in both the pre-HAART era and HAART era. In these studies, oral GCV at 1000 mg thrice daily was used in the pre-HAART era regimen [9,10] while VGCV at 900 mg twice daily was the regimen used in the HAART era [11]. The patients investigated in the above three studies were HIV-treatment-experienced patients. One study in the pre-HAART era reported the efficacy of preemptive therapy in patients with CD4 count<50 μl [9], while the other studies showed no significant preventive effect [10,11]. In the ACTG A5030 study, the prospective clinical trial in the HAART era, which evaluated the efficacy of oral VGCV 900 mg twice a day for 3 weeks among HIV-infected patients with CD4 count <100 cells/mm^3, plasma HIV RNA >400 copies/mL, plasma CMV viremia and on stable

Table 3. Results of multivariate analysis to estimate the preventive effect of CMV preemptive therapy against CMV end-organ disease.

	Model 1 Crude		Model 2 Adjusted		Model 3 Adjusted	
	HR	95% CI	HR	95%CI	HR	95%CI
CMV preemptive therapy*	0.286	0.087–0.939	0.289	0.088–0.949	0.172	0.049–0.602
Age			0.982	0.952–1.014	0.990	0.958–1.022
Female			1.033	0.310–3.441	0.988	0.267–3.653
CD4 count per 1/μl decrement					0.995	0.983–1.008
HIV viral load per log10/ml					2.217	0.912–5.393
CMV viral load per log10/ml*					1.941	1.266–2.975
Use of steroid					0.664	0.288–1.534
Chemotherapy					1.668	0.540–5.151
Concurrent AIDS					0.930	0.337–2.569

*P<0.05 in Model 3
HR: hazard ratio, CI: confidence interval
The Cox proportional hazards regression analysis was used.
Variables with significant difference by univariate analysis or assumed as risk factors for CMV-EOD in the literature were included in model 3.
doi:10.1371/journal.pone.0065348.t003

or no HAART, the authors reported a low incidence of CMV-EOD among subjects both with and without preemptive therapy [11]. The authors attributed the low incidence to improvement of immune function induced by potent ART. Actually, in that study [11], the number of patients who had received ART at study entry was about 80% of the total. In contrast, the subjects of our study were all treatment-naïve patients and possibly at higher risk for CMV-EOD compared to those enrolled in the ACTG A5030. Thus, the use of CMV preemptive therapy reported in our study under the clinical scenario of poor immune status without ART at study entry resulted in better outcome than in previous studies. In our study, there was no significant difference in the timing of ART between the two treatment groups. Although our study did not include the time to the initiation of ART as a variable in uni- and multivariate analysis because the values for 11 cases were missing, multivariate analysis with the time to the initiation of ART together with other variables similarly identified preemptive therapy as a significant preventive factor (adjusted HR = 0.235; 95%CI, 0.064–0.868; p = 0.030).

The survival benefits of CMV preemptive therapy were controversial in previous prospective clinical trials. One study suggested the survival benefits of 3 g/day oral GCV preemptive therapy [9], while other studies showed no evidence of the survival benefit [10]. On the other hand, two prospective cohort studies in the HAART era showed the relation between CMV viremia and high mortality [21] and suggested the benefit of CMV therapy [22], whereas our results showed no significant difference in mortality rate between the two groups. The reason for this discrepancy could be attributed to low mortality rate, small sample size and the disproportionally high risk of the therapy group in our study. The mortality rate (5.0 deaths per 100 person-years) in our study was similar to that in a study conducted in the HAART era (5.7 deaths per 100 person-years)[19] and was considerably lower than in studies from the pre-HAART era. Since the mortality rate has markedly decreased in advanced HIV infected patients following the introduction of potent ART in the HAART era [23,24], not only the survival benefit but also quality of life, such as improvement of eye function, should be emphasized in the future.

The side effects of preemptive therapy have also been of concern [25]. Our findings showed the development of grade 3 to 4 leukocytopenia in 23.3% of the patients who received intravenous GCV, and was the major side effect of preemptive therapy. Some patients who developed leukocytopenia required treatment with granulocyte colony-stimulating factor (G-CSF) and showed complete recovery. Thus; careful follow-up of patients on preemptive therapy is necessary. For these reasons, preemptive

Table 4. Details of CMV end-organ disease.

CMV-EOD	n (%)	Time to development (days)	Non-preemptive therapy group	Preemptive therapy group
Retinitis	22* (61.1%)	72 (14–326)	19* (57.6%)	3 (100%)
Esophagitis	4* (11.1%)	116.5(69–164)	4* (12.1%)	0
Gastroenteritis	3* (8.3%)	19 (14–40)	3* (9.1%)	0
Colitis	6* (16.7%)	40.5 (15–55)	6* (18.2%)	0
Pneumonitis	1 (2.8%)	31 (31–31)	1 (3.0%)	0
Total	36* (100%)	55 (14–326)	33* (100%)	3 (100%)

*Three patients of the non-preemptive therapy group had multiple CMV-EOD; one with retinitis plus esophagitis, one with retinitis plus gastroenteritis and the other with retinitis plus colitis.
doi:10.1371/journal.pone.0065348.t004

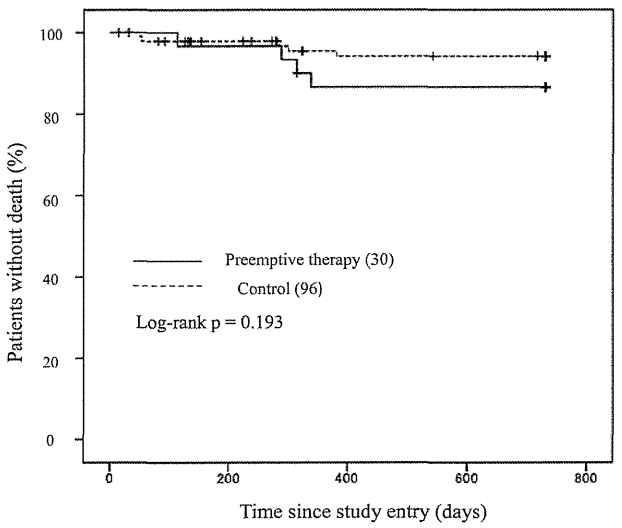


Figure 3. Kaplan-Meier curve showing the time to death in the preemptive and non-preemptive therapy groups. There was no significant difference in the survival rate between the two groups (p = 0.193, Log-rank test).
doi:10.1371/journal.pone.0065348.g003

therapy might place patients at greater risk in resource-limited setting, where close monitoring is difficult and the risk of bacterial infection is high. It is noteworthy, however, that death and bacterial infection related to preemptive therapy were not observed in our study.

The present study has several limitations. Due to its retrospective nature, it was not possible to control the baseline characteristics of the enrolled patients. However, patients with potential risk for CMV-EOD, such as those with high plasma CMV DNA, high concurrent AIDS and high steroid use, were more likely prescribed the preemptive therapy. It is noteworthy that the incidence of CMV-EOD was significantly lower in the preemptive therapy group despite this adverse environment.

Second, the criteria for treatment, choice of drugs and duration of CMV preemptive therapy were not rigidly controlled in the present study. Thus, it was difficult to determine which anti-CMV agent with what dosage is optimal for preemptive therapy. In the present study, about 90% of patients received induction dose or maintenance dose of GCV since the majority of patients of the preemptive therapy group were in-patients. Further prospective study is required to optimize effective preemptive therapy, including oral VGCV.

Third, CMV-EOD, especially enteritis, could have been overlooked at study entry since routine endoscopic screening was not performed, compared with screening for retinitis at the first visit. However, patients with abdominal pain were subjected to stool examination for occult blood, since the definition of CMV enteritis includes abdominal pain, and those with positive tests were subsequently considered for endoscopy. Thus, the possibility of latent CMV enteritis at study entry does not seem to have affected the results of the present study.

In conclusion, the present study demonstrated a lower incidence of CMV-EOD following CMV preemptive therapy by one-fourth, compared with no preemptive therapy, in treatment-naïve patients with CMV viremia. High plasma CMV DNA was identified as an independent risk for CMV-EOD. Further studies are warranted to elucidate the efficacy, safety and cost-effectiveness of anti-CMV preemptive therapy in HIV infected patients at high risk for EOD.

Supporting Information

Table S1 Definitions of CMV end-organ diseases used in this study.
(DOCX)

Acknowledgments

The authors thank all the clinical staff at the AIDS Clinical Center for their help in completion of this study.

Author Contributions

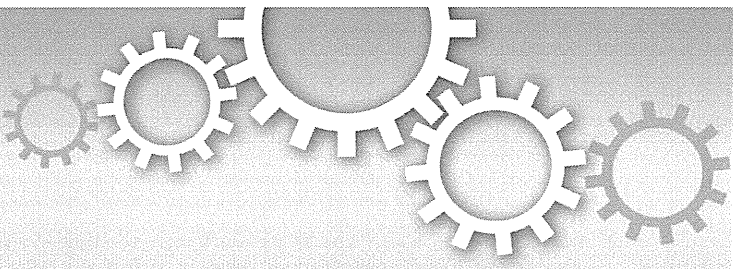
Conceived and designed the experiments: DM K. Tsukada K. Teruya. Performed the experiments: DM TN K. Teruya. Analyzed the data: DM HG YK SO. Contributed reagents/materials/analysis tools: YK K. Tsukada. Wrote the paper: DM TN HG SO.

References

1. Jabs DA, Van Natta ML, Holbrook JT, Kempen JH, Meinert CL, et al. (2007) Longitudinal study of the ocular complications of AIDS: 1. Ocular diagnoses at enrollment. Ophthalmology 114: 780–786.
2. Goodrich JM, Mori M, Gleaves CA, Du Mond C, Cays M, et al. (1991) Early treatment with ganciclovir to prevent cytomegalovirus disease after allogeneic bone marrow transplantation. N Engl J Med 325: 1601–1607.
3. Kalil AC, Levitsky J, Lyden E, Stoner J, Freifeld AG (2005) Meta-analysis: the efficacy of strategies to prevent organ disease by cytomegalovirus in solid organ transplant recipients. Ann Intern Med 143: 870–880.
4. Kalil AC, Freifeld AG, Lyden ER, Stoner JA (2009) Valganciclovir for cytomegalovirus prevention in solid organ transplant patients: an evidence-based reassessment of safety and efficacy. PLoS One 4: e5512.
5. Park JM, Lake KD, Arenas JD, Fontana RJ (2006) Efficacy and safety of low-dose valganciclovir in the prevention of cytomegalovirus disease in adult liver transplant recipients. Liver Transpl 12: 112–116.
6. Humar A, Kumar D, Preiksaitis J, Boivin G, Siegal D, et al. (2005) A trial of valganciclovir prophylaxis for cytomegalovirus prevention in lung transplant recipients. Am J Transplant 5: 1462–1468.
7. Kaplan JE, Benson C, Holmes KH, Brooks JT, Pau A, et al. (2009) Guidelines for prevention and treatment of opportunistic infections in HIV-infected adults and adolescents: recommendations from CDC, the National Institutes of Health, and the HIV Medicine Association of the Infectious Diseases Society of America. MMWR Recomm Rep 58: 1–207; quiz CE201-204.
8. Rose DN, Sacks HS (1997) Cost-effectiveness of cytomegalovirus (CMV) disease prevention in patients with AIDS: oral ganciclovir and CMV polymerase chain reaction testing. AIDS 11: 883–887.
9. Spector SA, McKinley GF, Lalezari JP, Samo T, Andruczk R, et al. (1996) Oral ganciclovir for the prevention of cytomegalovirus disease in persons with AIDS. Roche Cooperative Oral Ganciclovir Study Group. N Engl J Med 334: 1491–1497.
10. Brosgart CL, Louis TA, Hillman DW, Craig CP, Alston B, et al. (1998) A randomized, placebo-controlled trial of the safety and efficacy of oral ganciclovir for prophylaxis of cytomegalovirus disease in HIV-infected individuals. Terry Beirn Community Programs for Clinical Research on AIDS. AIDS 12: 269–277.
11. Wohl DA, Kendall MA, Andersen J, Crumpacker C, Spector SA, et al. (2009) Low rate of CMV end-organ disease in HIV-infected patients despite low CD4+ cell counts and CMV viremia: results of ACTG protocol A5030. HIV Clin Trials 10: 143–152.
12. Spector SA, Wong R, Hsia K, Pilcher M, Stempien MJ (1998) Plasma cytomegalovirus (CMV) DNA load predicts CMV disease and survival in AIDS patients. J Clin Invest 101: 497–502.
13. Spector SA, Hsia K, Crager M, Pilcher M, Cabral S, et al. (1999) Cytomegalovirus (CMV) DNA load is an independent predictor of CMV disease and survival in advanced AIDS. J Virol 73: 7027–7030.
14. Pergam SA, Xie H, Sandhu R, Pollack M, Smith J, et al. (2012) Efficiency and Risk Factors for CMV Transmission in Seronegative Hematopoietic Stem Cell Recipients. Biol Blood Marrow Transplant.
15. Kute VB, Vanikar AV, Shah PR, Gumber MR, Patel HV, et al. (2012) Post-renal transplant cytomegalovirus infection: study of risk factors. Transplant Proc 44: 706–709.
16. Fielding K, Koba A, Grant AD, Charalambous S, Day J, et al. (2011) Cytomegalovirus viremia as a risk factor for mortality prior to antiretroviral therapy among HIV-infected gold miners in South Africa. PLoS One 6: e25571.

17. Micol R, Buchy P, Guerrier G, Duong V, Ferradini L, et al. (2009) Prevalence, risk factors, and impact on outcome of cytomegalovirus replication in serum of Cambodian HIV-infected patients (2004-2007). J Acquir Immune Defic Syndr 51: 486–491.
18. Yoshida A, Hitomi S, Fukui T, Endo H, Morisawa Y, et al. (2001) Diagnosis and monitoring of human cytomegalovirus diseases in patients with human immunodeficiency virus infection by use of a real-time PCR assay. Clin Infect Dis 33: 1756–1761.
19. Erice A, Tierney C, Hirsch M, Caliendo AM, Weinberg A, et al. (2003) Cytomegalovirus (CMV) and human immunodeficiency virus (HIV) burden, CMV end-organ disease, and survival in subjects with advanced HIV infection (AIDS Clinical Trials Group Protocol 360). Clin Infect Dis 37: 567–578.
20. Hodge WG, Boivin JF, Shapiro SH, Shah KC, Dionne MA (2005) Iatrogenic risk factors for cytomegalovirus retinitis. Can J Ophthalmol 40: 701–710.
21. Deayton JR, Prof Sabin CA, Johnson MA, Emery VC, Wilson P, et al. (2004) Importance of cytomegalovirus viraemia in risk of disease progression and death in HIV-infected patients receiving highly active antiretroviral therapy. Lancet 363: 2116–2121.
22. Kempen JH, Martin BK, Wu AW, Barron B, Thorne JE, et al. (2003) The effect of cytomegalovirus retinitis on the quality of life of patients with AIDS in the era of highly active antiretroviral therapy. Ophthalmology 110: 987–995.
23. Ray M, Logan R, Sterne JA, Hernandez-Diaz S, Robins JM, et al. (2010) The effect of combined antiretroviral therapy on the overall mortality of HIV-infected individuals. AIDS 24: 123–137.
24. Sterne JA, May M, Costagliola D, de Wolf F, Phillips AN, et al. (2009) Timing of initiation of antiretroviral therapy in AIDS-free HIV-1-infected patients: a collaborative analysis of 18 HIV cohort studies. Lancet 373: 1352–1363.
25. Biron KK (2006) Antiviral drugs for cytomegalovirus diseases. Antiviral Res 71: 154–163.

SCIENTIFIC REPORTS



OPEN

SUBJECT AREAS:
INFECTION
HIV INFECTIONS
RETROVIRUS
VIRAL MEMBRANE FUSION

Received
12 April 2013

Accepted
24 July 2013

Published
8 August 2013

Correspondence and requests for materials should be addressed to H.G. (higatana@acc.ncgm.go.jp)

Arginine insertion and loss of N-linked glycosylation site in HIV-1 envelope V3 region confer CXCR4-tropism

Kiyoto Tsuchiya[1], Hirotaka Ode[2,3], Tsunefusa Hayashida[1,4], Junko Kakizawa[1], Hironori Sato[2], Shinichi Oka[1,4] & Hiroyuki Gatanaga[1,4]

[1]AIDS Clinical Center, National Center for Global Health and Medicine, 1-21-1 Toyama, Shinjuku-ku, Tokyo 162-8655, Japan, [2]Pathogen Genomics Center, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashimurayama-shi, Tokyo 208-0011, Japan, [3]Clinical Research Center, National Hospital Organization Nagoya Medical Center, 4-1-1 Sannomaru, Naka-ku, Nagoya 460-0001, Japan, [4]Center for AIDS Research, Kumamoto University, 2-2-1 Honjo, Chuo-ku, Kumamoto 860-0811, Japan.

The third variable region (V3) of HIV-1 envelope glycoprotein gp120 plays a key role in determination of viral coreceptor usage (tropism). However, which combinations of mutations in V3 confer a tropism shift is still unclear. A unique pattern of mutations in antiretroviral therapy-naive HIV-1 patient was observed associated with the HIV-1 tropism shift CCR5 to CXCR4. The insertion of arginine at position 11 and the loss of the N-linked glycosylation site were indispensable for acquiring pure CXCR4-tropism, which were confirmed by cell-cell fusion assay and phenotype analysis of recombinant HIV-1 variants. The same pattern of mutations in V3 and the associated tropism shift were identified in two of 53 other patients (3.8%) with CD4$^+$ cell count <200/mm^3. The combination of arginine insertion and loss of N-linked glycosylation site usually confers CXCR4-tropism. Awareness of this rule will help to confirm the tropism prediction from V3 sequences by conventional rules.

Since the introduction of maraviroc, a specific CCR5 antagonist, into clinical practice, scientific and clinical studies have focused on the coreceptor usage of human immunodeficiency virus type 1 (HIV-1)[1]. Evidence indicates the presence of a homogeneous population of predominantly CCR5-tropic variants[2,3] early in HIV-1 infection[4,5]. CXCR4-tropic variants[6,7], against which specific CCR5 antagonists are inefficient, can be distinguished from R5-tropic variants by their tendency for higher replication kinetics and a broader target cell range[8]. Their presence *in vivo* has been associated with accelerated fall in CD4$^+$ cell count and rapid disease progression[9,10]. CXCR4-tropic variants evolve from CCR5-tropic ones in the natural course of HIV-1 infection, though the exact mechanism of viral tropism evolution is not known yet. Long-term observation of natural course, which is indispensable for understanding the mechanism of tropism evolution, is usually difficult, because early use of antiretroviral therapy (ART) is highly recommended[11]. In this study, untreated natural course of one hemophiliac, who acquired HIV-1 infection through contaminated blood product before 1985 and exhibited slow disease progression, was followed until a rapid fall in CD4$^+$ cell count in 2007. The sequence change in the HIV-1 envelope (Env) glycoprotein gp120 V3 region (V3), the main determinant of HIV-1 tropism[12], was analyzed between 2003 and 2007. The results identified a unique change in 2007 associated with change in HIV-1 tropism. The same kind of sequence change in V3 was also identified in two other patients and in some of the registered sequences in the Los Alamos HIV sequence database.

Results

V3 sequence changes in Case 1. Case 1 was an ART-naive Japanese hemophiliac who acquired HIV-1 subtype B infection through contaminated blood product before 1985 and exhibited a slow disease progression. We reported previously the emergence of an escape mutation in HIV-1 Pol from cytotoxic T-lymphocyte (CTL) response in association with a mild increase in viral load in 1999 in this patient (KI-127)[13]. The HIV-1 viral load was steady around 10^4 copies/mL between 2002 and 2006 (Figure 1a). However, at the end of 2006, the viral load began to increase, coupled with a rapid fall in CD4$^+$ cell count. Since the emergence of CXCR4-tropic variants was suspected, changes in the V3 region were analyzed at five time points (June 2003, April 2006, and January, April, and November 2007) by sequencing 19–27 clones. Originally, most of the clones had identical or resembled V3

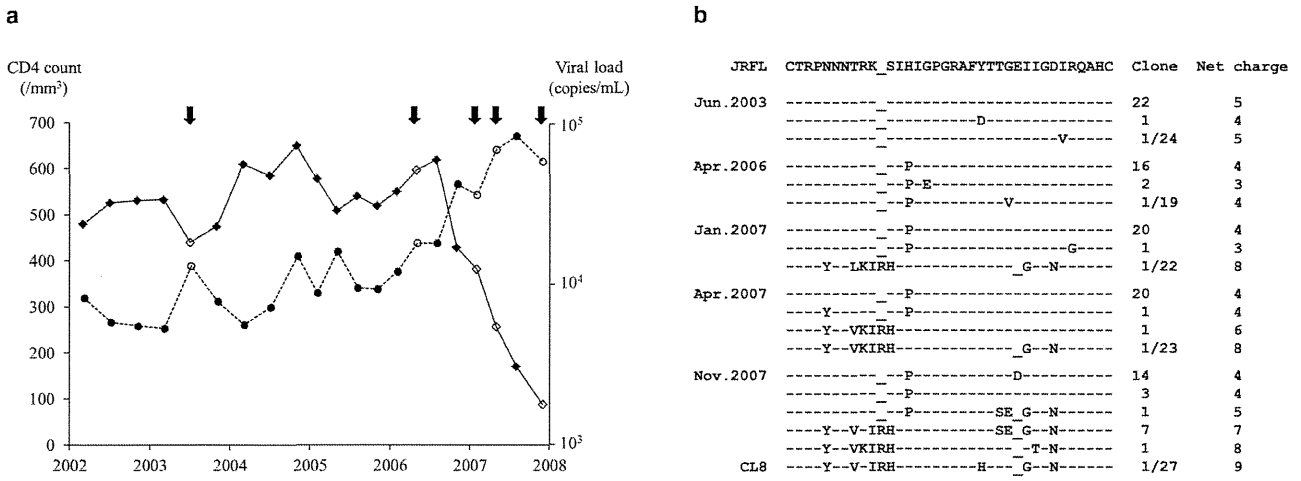


Figure 1 | (a). Clinical course of Case 1. The CD4$^+$ cell count (diamonds and solid lines) and HIV-1 load (circles and broken lines) from 2002 to 2008 are plotted. Arrows at the top indicate the five time points when V3 sequences were analyzed. Open diamonds and circles indicate the CD4$^+$ cell counts and HIV-1 loads at the same five time points. (b). V3 sequence changes in Case 1.Cloned sequences analyzed at the five time points are shown. The V3 sequence of HIV-1 JRFL is shown at the top column as a reference. Amino acids identical to those of HIV-1 JRFL are indicated as dashes. The numbers of clones harboring the corresponding V3 sequences are shown on the right.

sequences with CCR5-tropic HIV-1 JRFL (Figure 1b). Interestingly, a unique clone, harboring arginine insertion at position 11 of V3 (Ins11R), one amino acid deletion at position 25 (Del25), and other multiple amino acid substitutions, was identified at a frequency of 1/22 in January 2007, and the frequency of the same kind of the clones subsequently increased to 2/23 in April 2007, and 9/27 in November 2007, which was considered to be associated with the rapid fall in CD4$^+$ cell count.

Insertion and deletion confer CXCR4-tropism. In the next step, cell-cell fusion assay was performed to analyze the effect of the observed V3 changes on viral tropism, using Env-expressing 293 T cells and CD4$^+$ and CCR5$^+$/CXCR4$^+$ COS-7 cells. One V3 clone harboring Ins11R and Del25 identified in November 2007, named CL8-V3 (Figure 1b), was incorporated into JRFL Env-expressing plasmid. The cell-cell fusion assay demonstrated that CL8-V3 was purely CXCR4-tropic whereas JRFL was purely CCR5-tropic (Figure 2a). Ins11R occurred by the insertion of 'ACA' between 'G' and 'T' of 'AGT' at position 11 at nucleotide sequence level, and therefore, the substitution of serine (S) with histidine (H) at position 12 (S12H) was also associated with Ins11R in Case 1 ('AGT' → 'AGACAT' at nucleotide level; 'S' → 'RH' at amino acid level [Ins11R/S12H]). To identify the determinant of observed tropism change, the plasmids harboring Ins11R/S12H, Del25, and Ins11R/S12H/Del25 were also constructed using JRFL backbone. In the cell-cell fusion assay, Ins11R/S12H decreased CCR5-tropism of

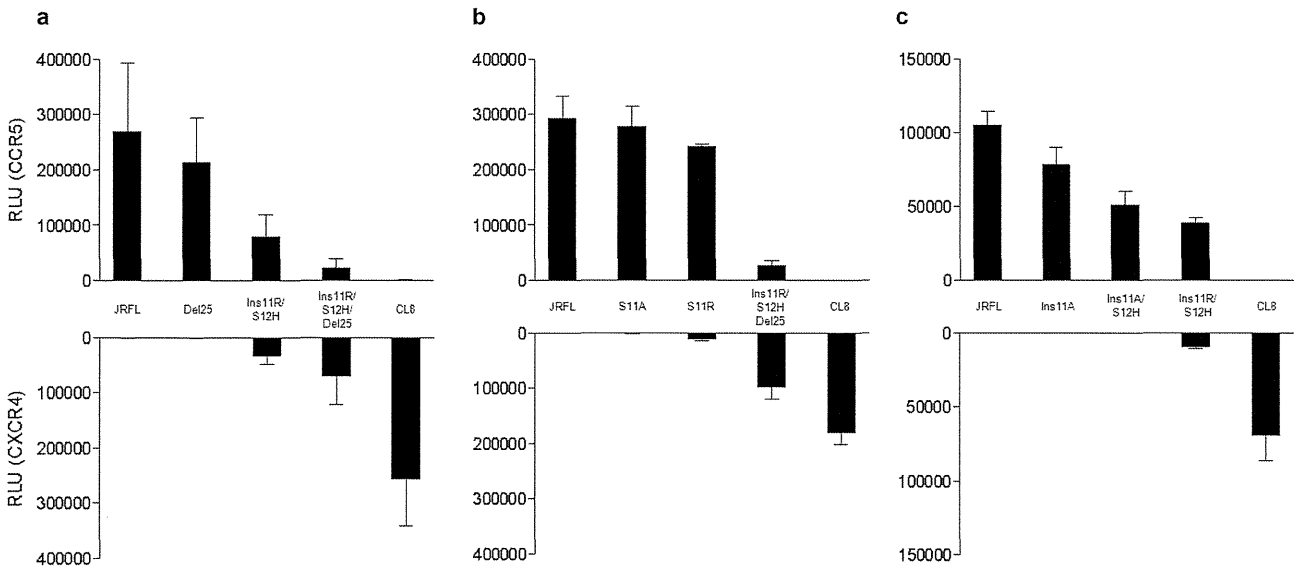


Figure 2 | Effect of Ins11R/S12H and Del25 in cell-cell fusion assay (a). Cell-cell fusion assay was performed using Env-expressing 293 T cells and CD4$^+$ and CCR5$^+$/CXCR4$^+$ COS-7 cells. Data are mean±SD values in relative luminescent unit (RLU) of six experiments (performed in duplicate and repeated three times). Analysis of two elements of Ins11R in cell-cell fusion assay (b and c). Effects of placing R at position 11 (b) and one amino acid elongation of V3 (c) were analyzed. Cell-cell fusion assay was performed using Env-expressing 293 T cells and CD4$^+$ and CCR5$^+$/CXCR4$^+$ COS-7 cells. Data are mean±SD values in relative luminescent unit (RLU) of six experiments (performed in duplicate and repeated three times).

JRFL-V3 and conferred CXCR4-tropism, resulting in dual-tropic (Ins11R/S12H vs. JRFL). Del25 further decreased the CCR5-tropism of Ins11R/S12H-V3 and increased CXCR4-tropism (Ins11R/S12H/Del25 vs Ins11R/S12H), though Del25 alone did not significantly change the JRFL-V3 tropism (Del25 vs JRFL). Considered together, the results suggest that Ins11R/S12H is indispensable for CXCR4-tropism of CL8-V3 and that Del25 strengthened the CXCR4-tropism in the presence of Ins11R/S12H. However, their combination was not enough to confer JRFL-V3 pure CXCR4-tropism (Ins11R/S12H/Del25-V3-expressing 293 T cells still fused with $CD4^+/CCR5^+$ COS-7 cells at low level), and some other mutations were necessary for pure CXCR4-tropism of CL8-V3.

Which is important, substitution or elongation? The above results suggested that Ins11R/S12H was indispensable for CXCR4-tropism of CL8-V3. According to the 11/25 rule, a basic amino acid residue (R or lysine [K]) at either position 11 or 25 of V3 is associated with CXCR4-tropism, whereas acidic or neutral amino acid residues at these positions are associated with CCR5-tropism[12,14,15]. Ins11R/S12H has two elements: one is to place R at position 11 and the other is one amino acid elongation of V3. To determine whether positioning R at 11 is sufficient for conferring CXCR4-tropism or whether V3 elongation is also necessary for this process, S at position 11 of JRFL-V3 was substituted with R (S11R) and alanine (A) (S11A) as reference. However, both substitutions did not alter the pure CCR5-tropism of JRFL (Figure 2b), indicating that not only R at position 11 but also one amino acid elongation was indispensable for dual tropism caused by Ins11R/S12H.

Is one amino acid elongation sufficient to induce CXCR4-tropism or is positioning R at 11 is also necessary? To answer this question, two V3-expressing plasmids were constructed; one harbored Ins11A only and the other harbored Ins11A and S12H (Ins11A/S12H). The cell-cell fusion assay indicated that Ins11A decreased and Ins11A/S12H further decreased infectivity with CCR5 though neither of them conferred CXCR4-tropism (Figure 2c). These results indicate that not only one amino acid elongation of V3 but also positioning R at 11 is indispensable for dual tropism caused by Ins11R/S12H.

Effect of net charge of V3. Ins11R/S12H conferred CXCR4-tropism and Del25 strengthened it. However, Ins11R/S12H/Del25-V3 was still dual-tropic, though CL8-V3 was purely CXCR-4 tropic. The next question was which mutation is necessary for Ins11R/S12H/Del25-V3 to become purely CXCR4-tropic, like CL8-V3 (to lose CCR5-tropism)? There are six amino acid substitutions in CL8-V3 (substitution of asparagine [N] with tyrosine [Y] at position 5 [N5Y], substitution of threonine [T] with valine [V] at position 8 [T8V], substitution of K with isoleucine [I] at position 10 [K10I], substitution of Y with H at position 22 [Y22H], substitution of V with glycine [G] at position 26 [V26G], and substitution of aspartic acid [D] with N at position 29 [D29N]), compared with Ins11R/S12H/Del25-V3. According to the net charge rule, the higher net charge of V3 is associated with CXCR4-tropism when calculated by subtracting the number of negatively charged amino acid residues (D and glutamic acid [E]) from the number of positively charged ones (K and R)[12,14]. D29N was the only amino acid substitution that increased the net charge of V3 among the six amino acid substitutions described above. Therefore, we analyzed the effect of D29N by adding D29N to Ins11R/S12H/Del25-V3 (Ins11R/S12H/Del25/D29N) and reverting it in CL8-V3 (CL8/N29D). In the cell-cell fusion assay, D29N reduced CCR5-tropism of Ins11R/S12H/Del25-V3 though it remained dual-tropic (Ins11R/S12H/Del25/D29N vs Ins11R/S12H/Del25), and the reversion of D29N did not change CL8-V3 tropism (CL8/N29D vs CL8) (see Supplementary Figure S1). These results indicate that D29N does not cause tropism difference between Ins11R/S12H/Del25-V3 and CL8-V3, indicating that the net charge rule did not work well.

***In silico* prediction of the effect of substitutions.** Adding D29N failed to alter the tropism of Ins11R/S12H/Del25-V3 from dual-tropic to purely CXCR4-tropic. There were five other amino acid substitutions between Ins11R/S12H/Del25-V3 and CL8-V3. Because the V3 conformation is important for coreceptor interactions[16] and because conformation of V3 loop is sensitive to V3 mutations[17,18], we examined how these V3 mutations could influence conformation of V3 in solution using molecular dynamics (MD) simulation[19]. In our MD simulation study, V3-loops of JRFL and Del25 (both CCR5-tropic) were placed in the opposite direction from the β20-β21 loop (Figure 3a), while CL8-V3 loop (CXCR4-tropic) was closed to and in the same direction with the β20-β21 loop (Figure 3c). The results were consistent with those obtained with $gp120_{LAI}$ recombinant outer domains containing CCR5-tropic and CXCR4-tropic V3 loop, respectively[17,18]. The loops of dual-tropic Ins11R/S12H-V3 and Ins11R/S12H/Del25 were located between Del25-V3 and CL8-V3 (Figure 3b). In fact, when the structural differences at the tip of the V3 tip region, i.e., the 'GPGR' amino acid residues were quantitatively measured with the root mean square deviation (RMSD) of the main chain[17], CL8-V3 was found to be located far from the loop of JRFL-V3 and Del25-V3, while those of Ins11R/S12H-V3 and Ins11R/S12H/Del25-V3 were between them (Table 1). These results suggest that our MD simulation could predict the V3 tropism based on the magnitude of the RMSD values of the V3 loop tip. In the next step, each of the six amino acid substitutions of CL8-V3 was incorporated into Ins11R/S12H/Del25-V3, and the location and conformation of the constructed loop was analyzed. When D29N was incorporated, the RMSD from JRFL-V3 decreased and that from CL8-V3 increased (Table 2), and the loop orientation was still similar to that of Ins11R/S12H/Del25 (Figure 3d), suggesting that D29N does not seem to change the tropism, compatible with the results of the cell-cell fusion assay (see Supplementary Figure S1). Among other single amino acid substitutions, only T8V was found to increase the RMSD from JRFL-V3 and decrease that from CL8-V3 (Table 2), and caused a positional shift of the V3 resembling that of the CL8-V3 (Figure 3e). N5Y did not change the orientation of the V3 loop (see Supplementary Figure S2a) though the RMSD from CL8-V3 increased and that from JRFL-V3 decreased (Table 2). K10I, Y22H, and V26G decreased the RMSD from JRFL-V3 and increased that from CL8-V3 (Table 2), and the V3 loop orientation was distinct from both Ins11R/S12H/Del25-V3 and CL8-V3 (see Supplementary Figure S2b, S2c, and S2d). These results suggest that among the six amino acid substitutions, T8V has the greatest impact on the tropism shift toward CXCR4-tropic.

Impact of T8V. Our *in silico* modeling predicted that T8V could alter the tropism of Ins11R/S12H/Del25-V3. In the next series of experiments, we incorporated T8V into JRFL-V3 (JRFL/T8V) and Ins11R/S12H/Del25-V3 (Ins11R/S12H/Del25/T8V), and analyzed the effect of such incorporation on their tropism using the cell-cell fusion assay. The incorporation of T8V into JRFL-V3 increased CCR5-tropism though it did not confer CXCR4-tropism (Figure 4a and 4b). However, T8V abrogated CCR5-tropism of Ins11R/S12H/Del25-V3 and converted it to purely CXCR4-tropic, although it did not increase CXCR4-tropism and Ins11R/S12H/Del25/T8V-V3 still had smaller CXCR4-tropism than CL8-V3 (Figure 4a). The combination of Ins11R/S12H/T8V was sufficient to confer CXCR4-tropism, although Del25/T8V did not (Figure 4b). T8V breaks the N-linked glycosylation motif 'NXT' at position 6–8 of V3, the loss of which was reported with tropism shift towards CXCR4-tropic[20,21]. Our results indicated that T8V was indispensable for pure CXCR4-tropism of CL8, which seemed to support the previous findings of the importance of the loss of N-linked glycosylation motif for CXCR4-tropism. The loss of the glycan moiety in V3 stem might lead to change gp120 interaction surface for coreceptor binding and influence coreceptor

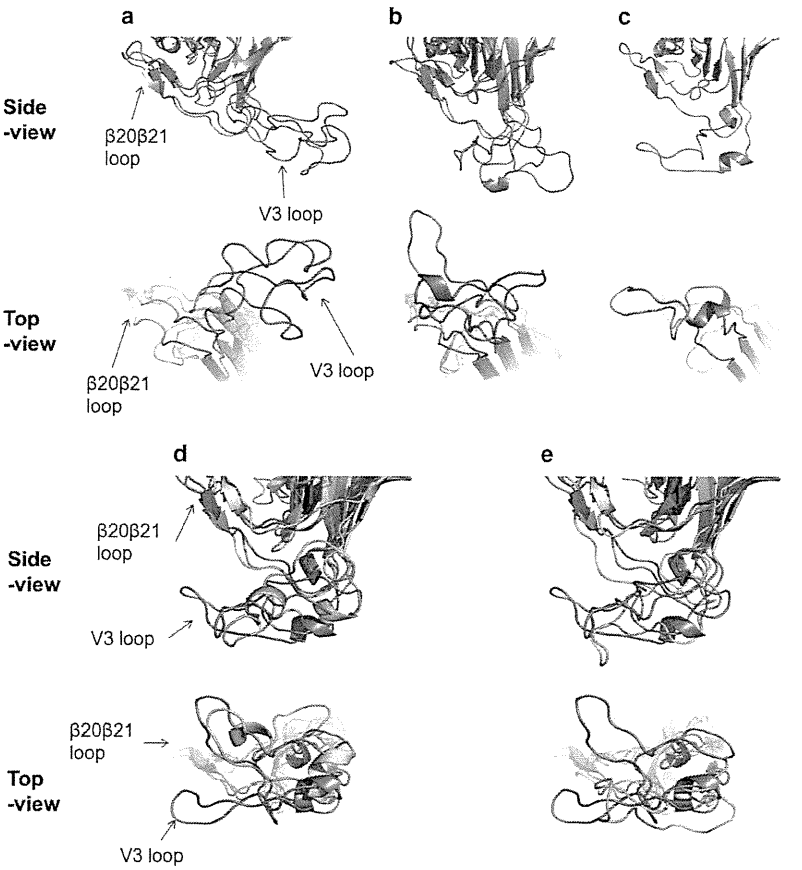


Figure 3 | Structural models of V3 loops on HIV-1 gp120 outer domains (a, b and c). MD simulations were performed for the HIV-1 JRFL gp120 outer domain with various V3 loops for CCR5 (a), dual (b), and CXCR4 (c) tropism. The most frequently appeared structures during 5–10 ns of MD simulations were extracted, and the top and side views of the structures around V3 loops are highlighted. (a) JRFL (gray) and Del25 (navy). (b) Ins11R/S12H/Del25 (gray) and Ins11R/S12H (navy). (c) CL8 (gray). Structural models of V3 loops of Ins11R/S12H/Del25-derived mutants (d and e). MD simulations were performed for the HIV-1 Ins11R/S12H/Del25 gp120 outer domain with D29N (d) or T8V (e) substitution in V3 loop. The most frequently appeared structures during 5–10 ns of MD simulations were extracted and superimposed with those of Ins11R/S12H/Del25 and CL8. (d) Superimposition of D29N (green), Ins11R/S12H/Del25 (gray), and CL8 (navy). (e) Superimposition of Ins11R/S12H/Del25/T8V (green), Ins11R/S12H/Del25 (gray), and CL8 (navy). Top and side views of the structures around V3 loops are shown.

tropism. However, available structural information was against this possibility, because the glycosylation site was exposed toward an opposite direction from the putative coreceptor binding site on V3[16,22,23]. Accordingly, presence or absence of the glycan moiety in V3 stem did not cause significant differences in V3 configuration in our MD simulation system[17,24]. Probably, amino acid substitution itself altered V3 configuration and coreceptor tropism.

Table 1 | Overall structural differences between the two V3 loop tips of various HIV-1 variants

	RMSD (Å)*			
ID of V3	JRFL	Del25	Ins11R/S12H	Ins11R/S12H/Del25
Del25	13.8	-	-	-
Ins11R/S12H	17.4	8.6	-	-
Ins11R/S12H/Del25	29.4	28.7	23.6	-
CL8	38.9	37.5	33.1	14.2

*RMSD values of the main chain atoms at V3 tips (GPGR) of two gp120 outer domain models from MD simulations. A smaller RMSD value means a closer conformation between two gp120s.

GHOST cell infection assay. Our cell-cell fusion assay indicated that Ins11R/S12H and T8V were indispensable for pure CXCR4-tropism of CL8. The next series of experiments were designed to confirm the findings using HIV-1 infection assay in GHOST cells[25,26]. HIV-1 JRFL and the recombinant HIV-1 variants harboring Del25-V3 and T8V-V3 had the same level of CCR5-tropism, although none could infect CXCR4$^+$ GHOST cells (Figure 4c). In comparison, Ins11R/S12H-V3- and Ins11R/S12H/Del25-V3-harboring variants had lower levels of CCR5-tropism. The latter variant, but not the former, infected CXCR4$^+$ GHOST cells though at low level. The Ins11R/S12H/Del25/T8V-V3-harboring variant lost the CCR5-tropism and acquired CXCR4-tropism, although the level of CXCR4-tropism was still lower than those of CL8-V3-harboring variant and HIV-1 NL4-3 (a CXCR4-tropic experimental strain). These results were compatible with the abovementioned results of the cell-cell fusion assay, though the CCR5-tropism of Ins11R/S12H/Del25-V3 seemed stronger in the cell infection assay. Dual-tropic Ins11R/S12H/Del25-V3 might have decreased susceptibility to AMD3100 used in the CCR5$^+$ GHOST Hi5 assay compared with pure CXCR4-tropic CL8-V3 and NL4-3-V3.

Same V3 pattern in two other cases. The analysis of V3 sequence changes in Case 1 demonstrated that Ins11R and the loss of N-linked

Table 2 | Effect of a single amino acid substitution on overall structure of the gp120 V3 tip

ID of V3	Added mutations*	RMSD (Å)¶ JRFL	RMSD (Å)¶ CL8
Ins11R/S12H/Del25	None	29.4	14.2
Ins11R/S12H/Del25/N5Y	N5Y	32.5	14.1
Ins11R/S12H/Del25/T8V	T8V	33.6	12.6
Ins11R/S12H/Del25/K10I	K10I	26.3	39.4
Ins11R/S12H/Del25/Y22H	Y22H	28.6	27.0
Ins11R/S12H/Del25/V26G	V26G	28.4	19.6
Ins11R/S12H/Del25/D29N	D29N	28.4	17.0

*Added amino acid substitution in the V3 loop of the Ins11R/S12H/Del25 gp120.
¶RMSD values of the main chain atoms at V3 tips (GPGR) of two gp120 outer domain models from MD simulations.

glycosylation site indispensably contribute to a shift toward CXCR4-tropism. To determine whether this finding was true only in Case 1 or could be generalized to other cases, HIV-1 subtype B V3 sequences were analyzed in 53 other treatment-naïve patients with $CD4^+$ cell count $< 200/mm^3$. The same pattern of mutations was identified in two cases (3.8%). In one case (Case 2), four of twenty analyzed sub-clones of V3 sequences harbored Ins11R associated with S12H, Del25, and N6A resulting in the loss of N-linked glycosylation site, compared with JRFL-V3 (Figure 5). In the other case (Case 3), three of twenty-two sub-clones harbored Ins11R associated with S11R, Del25, and T8V, resulting in the loss of N-linked glycosylation site. To delineate the tropism of the V3 abovementioned clones, two V3 clones in each case, one harboring Ins11R and the loss of N-linked glycosylation site (KF6 in Case 2, T16 in Case 3 [see Figure 5]) and the other harboring none of them (KF8 in Case 2, T02 in Case 3 [see Figure 5]), was incorporated into JRFL Env-expressing plasmid. As expected, cell-cell fusion assay indicated that the clones harboring Ins11R and the loss of N-linked glycosylation site (KF6 and T16) were purely CXCR4-tropic, although the clones harboring none of them (KF8 and T02) were purely CCR5-tropic (Figure 6a and 6b). The results of the GHOST cell infection assay using V3-incorporated HIV-1 JRFL (Figure 6c) were similar to those of the cell-cell fusion assay. Accordingly, it was concluded that the findings of the indispensability of Ins11R and the loss of N-linked glycosylation site for CXCR4-tropism were not only true in Case 1 but also in other cases.

Discussion

The phenotypic assay Trofile™ (Monogram Bioscience, South San Francisco, CA), which is based on recombinant virus technology, has been the most widely used diagnostic test for the detection of CXCR4-tropic HIV-1 variants[27]. However, this method has logistical and technical limitations that make it far from convenient as a diagnostic test in clinical practice. Genotypic methods based on V3 sequence represent a more feasible alternative[28] and are progressively replacing phenotypic assays, though their clinical use requires good genotypic-phenotypic correlations. The 11/25 rule and the net charge rule were proposed for the tropism prediction from V3 sequence[12,14,15], although they show only a moderate correlation with the results of phenotypic assays[12,15,28]. The results of specific genotypic tools, such as geno2pheno (Max-Planck Institute, Munich, Germany)[29,30] and position-specific scoring matrix (PSSM)[31,32] are comparable to those of phenotypic assays, suggesting that there should be some more genetic determinations for viral tropism. In this study, we successfully demonstrated two rules other than the 11/25 rule and the net charge rule on the association with CXCR4-tropic variants. One was that R insertion at position 11 of V3, not just placing R at position 11 but also one amino acid elongation, strongly shifted the HIV-1 tropism towards CXCR4-tropic. The other was that the loss of N-linked glycosylation site in V3 also shifted viral tropism towards CXCR4-tropic, which was previously described in some reports[20,21]. In the V3 analysis in our index case, R insertion at position 11 conferred dual-tropism to originally CCR5-tropic V3, and the loss of N-linked glycosylation site altered it totally CXCR4-tropic (see Supplementary Figure S3). We identified these mutation patterns not only in the index case but also in two other cases. When we surveyed V3 sequences with tropism confirmed by phenotypic assay registered at the Los Alamos HIV sequence database (Los Alamos National laboratory, Los Alamos, NM) (http://www.hiv.lanl.gov. as of September 25, 2012), 28 sequences had R insertion at position 11; 7 of 199 (3.5%) CXCR4-tropic, 14 of 513

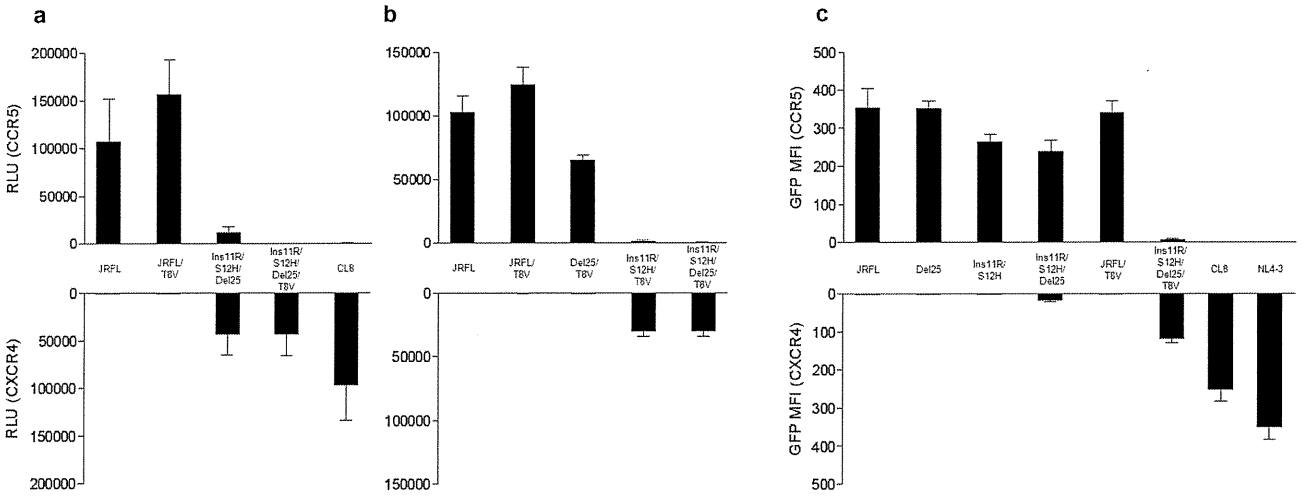


Figure 4 | Effects of T8V in cell-cell fusion assay (a and b). The effects of T8V were analyzed in combination with Ins11R/S12H/Del25 (a), and Del25 and Ins11R/S12H (b). Cell-cell fusion assay was performed using Env-expressing 293 T cells and $CD4^+$ and $CCR5^+$/$CXCR4^+$ COS-7 cells. Data are mean±SD values in relative luminescent unit (RLU) of six experiments (performed in duplicate and repeated three times). Tropism of recombinant HIV-1 variants harboring mutations identified in Case 1 (c). Tropism of HIV-1 variants was assessed in $CCR5^+$ GHOST Hi5 and $CXCR4^+$ GHOST CXCR4 cells. The mean fluorescent intensity (MFI) of infected cells expressing green fluorescent protein (GFP) was measured. Data are mean±SD values of six experiments (performed in duplicate and repeated three times).