b. 刊行物

(1) 木村哲；HIV/AIDS 25年の変遷－検査の促進に向けて．監訳・解説，2006

全訳 ① HIV/AIDSの25年－米国、1981～2006年．MMWR 55:585-589，2006

② カリニ肺炎－ロスアンジェルス．MMWR 30:250-252，1981

③ HIVの自宅検査．New Engl J Med 354(5):437-440，2006

④ 1つの疾患、2つの流行－25年目のAIDS．New Engl J Med 354(23):2411-2414，2006

⑤ 25年目のHIV/AIDSの世界的流行－世界の反応．New Engl J Med 354(23):2414-2417，2006

⑥ HIV抗体検査の価値観を変える－例外論の終り．New Engl J Med 355(7): 647-649，2006

解説 ① HIV/AIDS予防プログラムの進歩－1981～2006年アメリカ合衆国．MMWR 55:597-603，2006

② 若いMSMにおける最近のHIV検査：相関因子、背景、及びHIVセロコンバージョン．Sex Transm Dis 33:183-192，2006

③ MSMにおけるHIVの罹患率、認識されていない感染、HIV抗体検査－米国の5つの都市における調査（2004年6月～2005年4月）．MMWR 54:597-601，2005

④ 合衆国のおけるHIV感染認識者と非認識者による高いリスク性的行動についてのメタ解析 HIV予防プログラムへの影響．J Acquir Immune Defic Syndr 39:446-453，2005

⑤ HIVスクリーニングに関する米国予防医療専門委員会向けのエビデンスのレビュー．Ann Intern Med 143:55-73，2005

⑥ HIV感染の自宅検体採取検査．JAMA 280(19):1699-1701，1998

⑦ 医療施設における成人・青少年・妊婦を対象としたHIV検査実施の勧告－改訂版．MMWR 55 (RR-14)，2006

(2) 本田美和子，木村哲；医療機関における成人・若年者・妊婦のHIV検査に関する勧告改訂版「Revised Recommendations for HIV Testing of Adults, Adorescents, and Pregnant Women in Health-Care Settings」(MMWR 55 (No. RR14)：1-17，2006.9.22)．監訳・解説，2007

(3) 木村哲；医療保健施設での医療者主導によるHIV検査およびカウンセリングに関するガイダンス「Guidance on Provider-Initiated HIV Testing and Counselling in Healthcare Facilities」©World Heath Organization 2007．監訳，2009

BIOMEDICAL CHROMATOGRAPHY
Biomed. Chromatogr. **20**: 28–36 (2006)
Published online in Wiley InterScience (www.interscience.wiley.com).
DOI: 10.1002/bmc.521

ORIGINAL RESEARCH

Simultaneous determination of six HIV protease inhibitors (amprenavir, indinavir, lopinavir, nelfinavir, ritonavir and saquinavir), the active metabolite of nelfinavir (M8) and non-nucleoside reverse transcriptase inhibitor (efavirenz) in human plasma by high-performance liquid chromatography

Yoshihiro Hirabayashi, Kiyoto Tsuchiya, Satoshi Kimura and Shinichi Oka*

AIDS Clinical Center, International Medical Center of Japan, 1-21-1 Toyama, Shinjuku-ku, Tokyo 162-8655, Japan

Received 9 December 2004; revised 10 March 2005; accepted 15 March 2005

ABSTRACT: We report the development of a simple, economical and reliable chromatographic method for the simultaneous determination of six HIV protease inhibitors (PIs; amprenavir, indinavir, lopinavir, nelfinavir, ritonavir and saquinavir), the active metabolite of nelfinavir (M8) and the non-nucleoside reverse transcriptase inhibitor (NNRTI; efavirenz) in human plasma. After extraction from plasma with an ethyl acetate–acetonitrile mixture, the analytes were separated on a phenyl column with a gradient of acetonitrile and phosphate solutions, and detected at three ultraviolet wavelengths. Calibration curves were linear over the range 0.025–15 μg/mL for saquinavir and 0.05–15 μg/mL for the other analytes. The accuracies ranged from −6.9% to +7.6%, and the intra-assay and inter-assay precisions were <9.2 and <11.8%, respectively. Our method, covering most of the PIs and NNRTIs currently used, facilitates ready therapeutic drug monitoring in hospital laboratories. Copyright © 2005 John Wiley & Sons, Ltd.

KEYWORDS: protease inhibitors; efavirenz; HIV; therapeutic drug monitoring; high-performance liquid chromatography

INTRODUCTION

Currently, more than 17 anti-human immunodeficiency virus (HIV) drugs have been approved and are in clinical use in industrialized countries. To obtain optimal antiviral efficacy and to prevent viral drug resistance, these drugs are administered to patients in combination regimens, which are referred to as highly active antiretroviral therapy (HAART). Current standard HAART regimens consist of one or two protease inhibitors (PIs) or one non-nucleoside reverse transcriptase inhibitor (NNRTI), together with two nucleoside reverse transcriptase inhibitors (NRTIs; Richman, 2001; British HIV Association Writing Committee, 2003; The Panel on Clinical Practices for Treatment of HIV Infection, 2004; Yeni *et al.*, 2004), but more complex regimens are often needed because of treatment failure. Since the introduction of HAART in the late 1990s, the short-term prognosis of HIV infection has dramatically improved (Palella *et al.*, 1998). However, a large degree of inter-patient variability is observed in the efficacy and toxicity of HAART and still remains to be resolved.

This variability is at least in part attributed to the pharmacokinetics of the drugs, especially PIs and NNRTIs (Barry *et al.*, 1998; Acosta *et al.*, 1999; Marzolini *et al.*, 2001). Cytochrome P450 (CYP), by which PIs and NNRTIs are extensively metabolized, and P-glycoprotein, which plays an important role in transportation of these drugs at tissue and cellular levels, have genetic polymorphisms, leading to the inter-patient variability in pharmacokinetics (Fellay *et al.*, 2002). Furthermore, PIs and NNRTIs are both potent CYP inducers and inhibitors, and as a result, complicated and unpredictable pharmacokinetic interactions with co-administered drugs frequently occur (Dresser *et al.*, 2000; Gerber, 2000). To overcome

*Correspondence to: S. Oka, AIDS Clinical Center, International Medical Center of Japan, 1-21-1 Toyama, Shinjuku-ku, Tokyo 162-8655, Japan.
E-mail: oka@imcj.hosp.go.jp

Abbreviations used: APV, amprenavir; CYP, cytochrome P450; EFV, efavirenz; HAART, highly active antiretroviral therapy; IDV, indinavir; LPV, lopinavir; NFV, nelfinavir; NNRTI, non-nucleoside reverse transcriptase inhibitor; PI, protease inhibitor; RTV, ritonavir; SQV, saquinavir; TDM, therapeutic drug monitoring.

Contract/grant sponsor: Ministry of Health, Labour and Welfare of Japan; Contract/grant number: H15-15100101.
Contract/grant sponsor: Japanese Foundation for AIDS Prevention.
Contract/grant sponsor: Organization of Pharmaceutical Safety and Research; Contract/grant number: 01-4.

Published online 22 June 2005

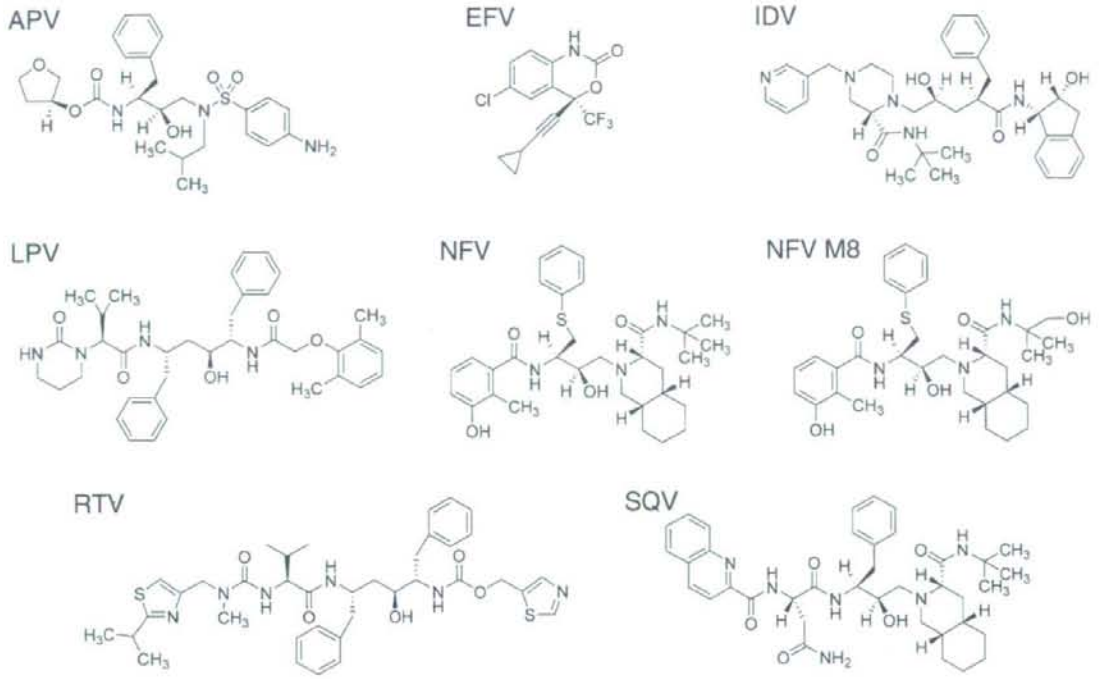

Figure 1. Chemical structures of APV, EFV, IDV, LPV, NFV, NFV M8, RTV and SQV.

the pharmacokinetic variability, therapeutic drug monitoring (TDM) for PIs and NNRTIs has recently been proposed as a practical and potential tool by clinicians (Acosta *et al.*, 2002; Back *et al.*, 2002; Van Heeswijk, 2002). In addition, TDM may be useful in assessing adherence to treatment, which is another clinical problem in HAART (Hugen *et al.*, 2002).

For the purpose of routine TDM in hospital laboratories, a simple and reliable analytical method that can simultaneously determine plasma concentrations of most PIs and NNRTIs is highly desirable. In this article, we describe a novel chromatographic method for the simultaneous determination of the six widely used PIs [amprenavir (APV), indinavir (IDV), lopinavir (LPV), nelfinavir (NFV), ritonavir (RTV) and saquinavir (SQV); Fig. 1] and a clinically important active metabolite of NFV, M8, together with efavirenz (EFV), which is the most frequently used NNRTI. Moreover, the usefulness of this method for TDM is discussed.

EXPERIMENTAL

Materials. NFV mesylate and its metabolite M8 were kindly provided by Japan Tobacco Inc. (Tokyo, Japan), IDV sulfate and EFV by Merck & Co. (Rahway, NJ, USA), RTV and LPV by Abbott Laboratories (Abbott Park, IL, USA), APV by Kissei Pharmaceutical Co. (Matsumoto City, Japan) and SQV mesylate by Roche Products (Welwyn Garden City, UK). The compounds tested for possible interference with the analytical method were obtained from Sigma-Aldrich Chemical Co. (St Louis, MO, USA) and Wako Pure Chemical Industries (Osaka, Japan), or were extracted from commercial products. Acetonitrile, methanol, distilled water (each of HPLC grade), disodium hydrogenphosphate (Na_2HPO_4), sodium dihydrogenphosphate (NaH_2PO_4) and sodium 1-hexanesulfonate were purchased from Nacalai Tesque Inc. (Kyoto, Japan). Ethyl acetate (HPLC grade), *n*-hexane (HPLC grade), ammonium hydroxide and phosphoric acid were obtained from Wako Pure Chemical Industries.

Drug-free blank plasma was collected from eight healthy volunteers in our hospital. Clinical samples were obtained from 10 HIV-infected patients receiving PIs and/or EFV as part of a HAART regimen. The Ethics Committee for the Clinical Study in our hospital approved this study (no. 39) and all participants provided written informed consent. The blood was drawn into heparinized tubes, and plasma was separated by centrifugation at 3000 ***g*** for 10 min and stored at −30°C.

Separate stock solutions of the eight analytes were prepared by dissolving the compound in methanol to yield a concentration of 1000 μg/mL as free base. Each solution was combined and diluted in 50% (v/v) methanol to give a working solution containing all eight analytes at 100 μg/mL. The working solution was further diluted in blank plasma for the preparation of calibration standards and quality controls.

Sample pretreatment. An aliquot of 400 μL plasma was transferred to a 5 mL glass tube and alkalinized with 400 μL of 0.1 M ammonium hydroxide adjusted to pH 10.5 with phosphoric acid. After vortexing briefly, 1.8 mL of an ethyl acetate–acetonitrile mixture (9:1, v/v), freshly prepared, was added to each tube. The tubes were vortexed vigorously at room temperature for 5 min and centrifuged at 2000 *g* at 4°C for 10 min. Then, 1.5 mL of the organic phase was transferred to a 2 mL tube and evaporated to dryness under a gentle stream of nitrogen at 40°C. Subsequently, the residue was reconstituted in 150 μL of solvent A (see 'Chromatography'). The resulting solution was washed with 1 mL *n*-hexane by vortexing for 10 s and centrifuging at 2000 *g* for 2 min. After eliminating the upper hexane layer, the non-hexane solution was filtrated through a 0.45 μm filter and transferred to an auto-sampler vial. Aliquots of 50 μL were injected into the HPLC unit.

Chromatography. Chromatography was performed using an integrated high-performance liquid chromatography (HPLC) unit, Integral 100Q (Applied Biosystems, Foster City, CA, USA), which consists of an automatic sample injector, a binary solvent delivery pump and a dual-wavelength ultraviolet detector. The analytical column was a Develosil Ph-UG-3 column (150 × 2.0 mm, 3 μm particle size; Nomura Chemical Co., Seto City, Japan) protected by a Develosil Ph-UG-S precolumn (10 × 1.5 mm; Nomura Chemical Co.). The temperature was maintained at 40°C using a Shodex column heater (Showa Denko Co., Tokyo, Japan). Analytical runs were processed by the Turbochrom software (version 3.01; Applied Biosystems).

The mobile phase consisted of a gradient of solvents A and B. Solvent A was a mixture of 34% (v/v) acetonitrile and 66% (v/v) 25 mM NaH_2PO_4 containing 6 mM sodium 1-hexanesulfonate and exactly adjusted to pH 5.1 with 25 mM Na_2HPO_4. Solvent B was composed of 64% (v/v) acetonitrile and 36% (v/v) 25 mM NaH_2PO_4 containing 6 mM sodium hexanesulfonate and adjusted to pH 5.3. Over the first 14.6 min of the run, 100% solvent A was delivered constantly, and then the proportion of solvent B was increased linearly from 0% to 30% over 2.0 min. From time 16.6 to 35.5 min, a mixture of 70% solvent A and 30% solvent B was used in an isocratic mode. The column was then rinsed with 100% solvent B for 4.5 min and semi-equilibrated with 100% solvent A for 6.8 min. The flow rate was maintained at 200 μL/min. Prior to use, solvents were passed through a 0.45 μm filter and degassed by helium sparging.

Absorbance was monitored at a dual wavelength: channel 1 was set at a wavelength of 212 nm through the run. For channel 2, the wavelength was set at 266 nm for the first 18.3 min, then switched to 239 nm automatically and switched back to 266 nm at the end of the run. APV, SQV and the other six analytes were detected at 266, 239 and 212 nm, respectively.

Method validation. The calibration was established over the range of 0.025–15 μg/mL for SQV and 0.05–15 μg/mL for the other analytes using seven spiked plasma samples. The calibration curve for each analyte was constructed by least-squares linear regression of the observed peak area vs the spiked concentration. Unknown concentrations of quality controls and patient samples were calculated from the linear regression equation of daily calibration curve for each analyte. The data analyses were performed using Turbochrom software (version 3.01; Applied Biosystems) on a Windows personal computer.

The accuracy and precision of the method were determined by assaying plasma samples spiked with the analytes at three different concentrations of 0.3, 2.1 and 12 μg/mL. Accuracy was defined as the percentage of deviation from the nominal concentration. Intra-assay precision was calculated as the relative standard deviation from six replicate quality controls within a single assay. Inter-assay precision was estimated from the analyses of quality controls on five separate days. The lower limits of quantitation (LLQs) were determined with plasma samples spiked with decreasing concentrations of the analytes (0.0125, 0.025, 0.05 and 0.1 μg/mL). The LLQ was defined as the lowest concentration for which the percentage deviation from the nominal concentration and the relative standard deviation were both less than 20%. The LLQ values were used for the lowest concentration of daily calibration curves. The upper limit of quantitation (ULQ) was arbitrarily determined as 15 μg/mL for each analyte.

The efficiency of the extraction procedure was determined with plasma samples spiked at three different concentrations of 0.3, 2.1 and 12 μg/mL in triplicate. The average recovery of each analyte was calculated by comparing the observed peak areas of the processed samples with those of non-processed standard solutions in mobile phase (solvent A). The stability of the analytes in plasma samples was investigated for three different conditions. Plasma was spiked with two concentrations of the analytes (0.3 and 12 μg/mL) and subsequently kept at 60°C for 60 min, 4°C for 7 days and −30°C for 60 days including three freeze–thaw cycles. These samples were analyzed together with freshly prepared samples and the ratios of the observed concentrations were calculated.

RESULTS

Representative chromatograms of plasma spiked with 1.8 μg/mL of each of the six PIs, NFV M8 and EFV are shown in Fig. 2. IDV, NFV M8, EFV, RTV, LPV and NFV were detected at a wavelength of 212 nm [Fig. 2(A)], based on their ultraviolet absorption spectra (data not shown). APV and SQV were monitored at 266 and 239 nm, respectively [Fig. 2(B)], because interfering endogenous peaks were observed near the peaks of these analytes at 212 nm. The retention times were 14.4, 16.2, 21.8, 23.7, 25.8, 30.0, 31.4 and 33.0 min for IDV, APV, NFV M8, EFV, SQV, RTV, LPV and NFV, respectively.

Blank plasma samples from eight healthy individuals showed no endogenous peaks interfering with any analyte. The typical chromatograms are presented in Fig. 3. A total of 35 drugs, which are frequently co-administered to HIV-infected patients, were also examined for possible analytical interference (Table 1). All the drugs but ketoconazole were eluted at retention times of <10 min or were not detected with the method. Ketoconazole had a retention time of 20.2 min,

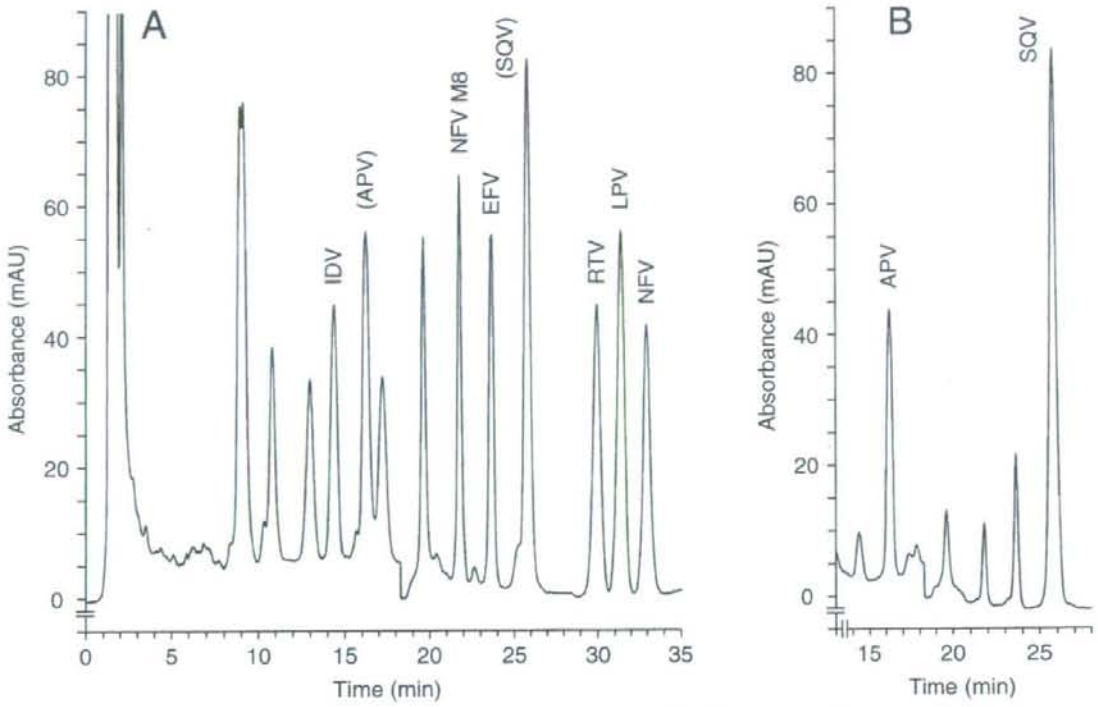

Figure 2. Chromatograms of a plasma sample spiked with 1.8 μg/mL of APV, EFV, IDV, LPV, NFV, NFV M8, RTV and SQV. Absorbance was monitored at 212 nm (A) and 266/239 nm (switched from 266 to 239 nm at 18.3 min) (B).

Table 1. Drugs tested for possible analytical interference

Abacavir	Erythromycin	Pentamidine
Acetaminophen	Ethambutol	Prednisolone
Aciclovir	Fluconazole	Pyrazinamide
Amikacin	Foscarnet	Rifampicin
Amoxicillin	Ganciclovir	Stavudine
Amphotericin B	Hydroxyurea	Sulfamethoxazole
Ampicillin	Isoniazid	Trimethoprim
Azithromycin	Kanamycin	Vancomycin
Cefaclor	Ketoconazole	Zalcitabine
Clarithromycin	Lamivudine	Zidovudine
Clindamycin	Metronidazole	Zidovudine glucuronide
Didanosine	Nevirapine	

which was obviously different from that of NFV M8 (21.8 min). Interference with metabolites of PIs and EFV was investigated with clinical samples, because these metabolites except NFV M8 are not available in pure form. No peaks interfering with any analyte were observed in plasma samples from patients receiving PIs and EFV (data not shown).

Over the concentration range 0.025–15 μg/mL for SQV and 0.05–15 μg/mL for the other seven analytes, the calibration curves were constructed by least-squares analysis. The correlation coefficients (r^2) of the curves were 0.995, 0.992, 0.998, 0.998, 0.997, 0.999, 0.998 and 0.999 for APV, EFV, IDV, LPV, NFV, NFV M8, RTV and SQV, respectively. The results of the accuracy and precision of the method are summarized in Table 2. The accuracies for the analytes at three concentration levels ranged from −6.9 to +7.6%. The intra-assay and inter-assay precisions were <9.2 and <11.8%, respectively. The LLQs were 0.025 μg/mL for SQV and 0.05 μg/mL for the other analytes, as determined with the plasma samples spiked with decreasing concentrations of the analytes. The ULQ was arbitrarily defined as 15 μg/mL for each analyte. The accuracies and precisions at the ULQ were also <20%.

The average extraction recoveries were 84.8, 70.9, 90.6, 88.3, 73.7, 80.0, 93.1 and 95.4% for APV, EFV, IDV, LPV, NFV, NFV M8, RTV and SQV, respectively. Although the reasons for the relatively lower recoveries of EFV and NFV are unclear, these had no negative effects on the assay performance as described above. The stability of the analytes in plasma samples is shown in Table 3. Under all conditions tested, the analytes proved to be stable with a recovery of >90.6% of the initial concentration.

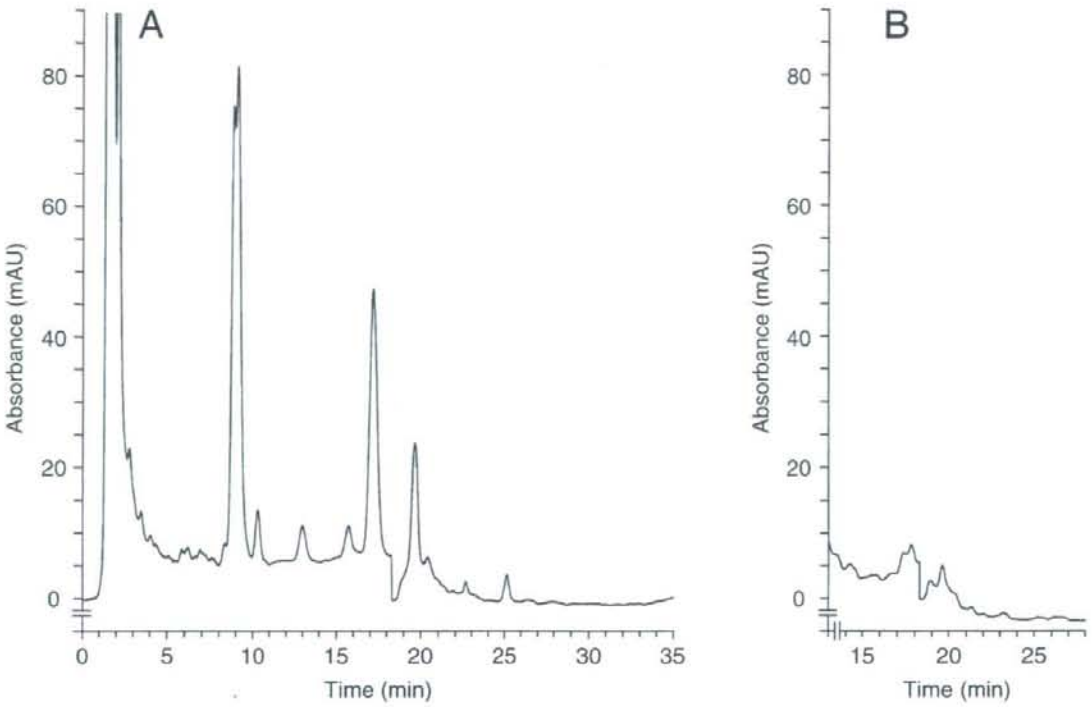

Figure 3. Chromatograms of a blank plasma sample. Absorbance was monitored at 212 nm (A) and 266/239 nm (switched from 266 to 239 nm at 18.3 min) (B).

Table 2. Accuracy and precision of the analytical method

Analyte	Concentration (μg/mL)	Accuracy (%)[a] ($n = 6$)	Precision (%)[b] Intra-assay ($n = 6$)	Precision (%)[b] Inter-assay ($n = 5$)
Amprenavir	0.3	+1.3	8.9	7.8
	2.1	−6.9	4.0	5.1
	12.0	+2.0	3.9	6.7
Efavirenz	0.3	+7.6	7.5	11.8
	2.1	−0.3	4.1	8.4
	12.0	+3.5	2.2	5.3
Indinavir	0.3	−2.0	6.1	9.0
	2.1	+1.7	3.1	4.9
	12.0	+5.2	5.4	4.3
Lopinavir	0.3	+4.5	5.6	9.5
	2.1	+2.8	4.0	6.1
	12.0	−0.9	4.3	5.9
Nelfinavir	0.3	+3.1	5.0	5.3
	2.1	+1.9	5.4	4.7
	12.0	−1.6	2.1	3.2
Nelfinavir M8	0.3	+4.4	4.6	6.7
	2.1	−0.3	2.9	4.4
	12.0	−1.6	1.5	3.6
Ritonavir	0.3	−2.0	9.2	7.3
	2.1	+3.2	3.8	5.1
	12.0	−4.1	3.1	5.9
Saquinavir	0.3	+1.7	3.9	2.8
	2.1	−0.2	2.2	3.4
	12.0	+3.1	4.2	4.7

[a] Deviation from nominal concentration.
[b] Relative standard deviation within a single assay or between different assays.

Table 3. Stability of protease inhibitors and efavirenz in plasma samples

Analyte	Concentration (μg/mL)	Recovery (%)[a] 60 min at 60°C (n = 3)	7 days at 4°C (n = 3)	60 days at −30°C with three freeze–thaw cycles (n = 4)
Amprenavir	0.3	94.7	104.8	95.6
	12.0	91.4	103.0	101.7
Efavirenz	0.3	95.8	100.7	98.8
	12.0	90.6	99.1	95.9
Indinavir	0.3	91.5	105.2	94.8
	12.0	93.2	100.8	98.6
Lopinavir	0.3	94.0	104.1	98.0
	12.0	93.3	101.3	100.5
Nelfinavir	0.3	102.4	102.5	99.8
	12.0	96.2	101.8	99.2
Nelfinavir M8	0.3	95.6	98.6	93.3
	12.0	98.1	101.8	97.6
Ritonavir	0.3	96.7	107.3	96.9
	12.0	93.2	103.0	97.2
Saquinavir	0.3	97.3	102.9	97.8
	12.0	92.8	102.6	100.4

[a] Compared with freshly prepared samples.

Table 4. Plasma concentrations of protease inhibitors and efavirenz in 10 patients

Patient no.	Drug	Dose (mg)[a]	Time after dosing (h)	Concentration (μg/mL)
1	Amprenavir	1200, b.i.d.	10.0	0.22
	Efavirenz	600, q.d.	10.0	0.53
2	Amprenavir	1200, b.i.d.	2.0	6.47
	Ritonavir	200, b.i.d.	2.0	0.38
	Efavirenz	600, q.d.	12.0	1.47
3	Indinavir	800, t.i.d.	6.5	0.19
4	Indinavir	400, b.i.d.	2.0	3.53
	Ritonavir	400, b.i.d.	2.0	2.55
5	Lopinavir	400, b.i.d.	3.0	5.94
	Ritonavir	100, b.i.d.	3.0	0.42
6	Lopinavir	400, b.i.d.	12.0	3.06
	Ritonavir	100, b.i.d.	12.0	0.09
7	Nelfinavir	1250, b.i.d.	4.5	1.01 (nelfinavir M8 0.96)
8	Nelfinavir	1250, b.i.d.	11.0	0.78 (nelfinavir M8 0.23)
9	Saquinavir	400, b.i.d.	3.5	1.49
	Ritonavir	400, b.i.d.	3.5	6.03
10	Saquinavir	400, b.i.d.	11.5	0.56
	Ritonavir	400, b.i.d.	11.5	1.30

[a] b.i.d., twice a day; q.d., once a day; t.i.d., three times a day.

The present method was applied to the determination of drug concentrations in clinical samples (Table 4 and Fig. 4). Plasma samples were randomly collected from 10 patients receiving PIs and EFV. All the samples could be analyzed with no technical difficulties. The observed concentrations were within the concentration ranges reported in literature (Khoo *et al.*, 2001; Acosta *et al.*, 2002; Back *et al.*, 2002; Van Heeswijk, 2002).

DISCUSSION

In this report, we describe the development, validation and clinical application of a novel chromatographic method for the simultaneous determination of the six widely used PIs and the most frequently used NNRTI, EFV, in plasma. To date, several methods have been reported for the simultaneous determination of different PIs or PIs plus NNRTIs (Leibenguth *et al.*, 2001;

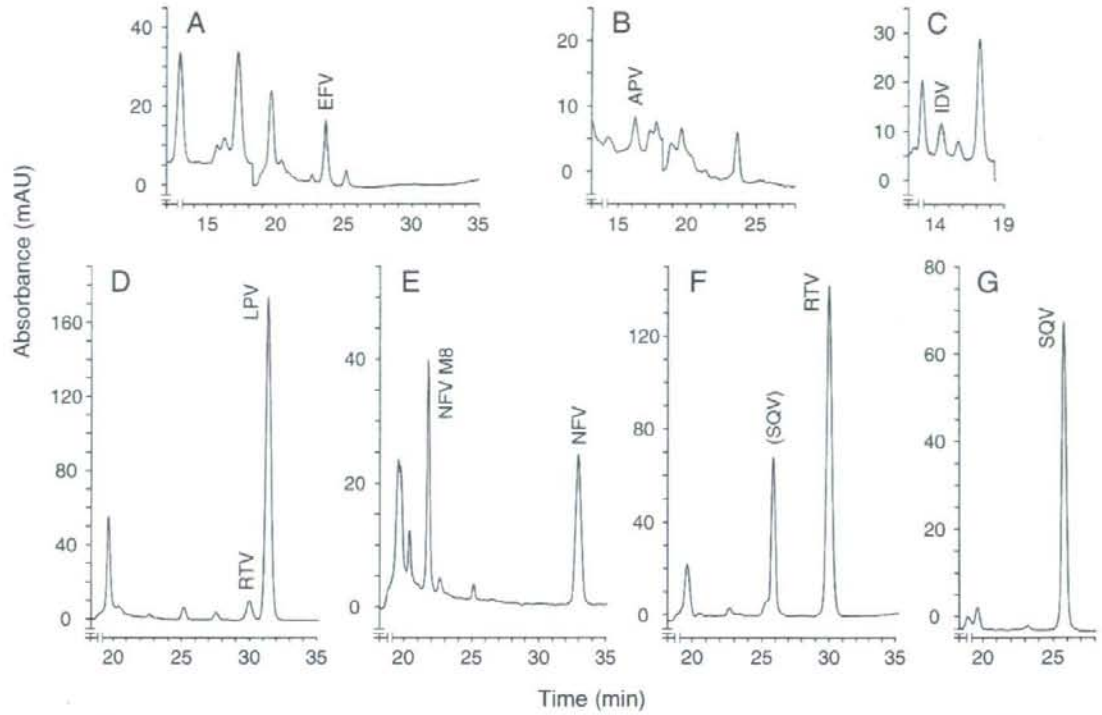

Figure 4. Chromatograms of plasma samples from patient 1 (A and B), patient 3 (C), patient 5 (D), patient 7 (E) and patient 9 (F and G). Absorbance was monitored at 212 nm (A, C, D, E and F) and 266/239 nm (switched from 266 to 239 nm at 18.3 min) (B and G). For details of the patients, see Table 4.

Poirier *et al.*, 2002; Titier *et al.*, 2002; Tribut *et al.*, 2002; Volosov *et al.*, 2002; Crommentuyn *et al.*, 2003; Frerichs *et al.*, 2003; Rentsch, 2003; Turner *et al.*, 2003; Rezk *et al.*, 2004). However, most of these methods have limitations in clinical application, including insufficient quantitation sensitivity, laborious sample pretreatment with solid-phase extraction, or use of expensive mass spectrometry. A simple, economical and reliable method that is performable with standard hospital laboratory equipment is desirable for routine TDM. The present method proved sufficiently sensitive to be used for TDM, because the LLQ values of the method for individual drugs were lower than the trough concentrations observed with treated patients and the target trough concentrations estimated from clinical and *in vitro* data (Khoo *et al.*, 2001; Acosta *et al.*, 2002; Back *et al.*, 2002; Van Heeswijk, 2002). The method was also accurate and precise over a wide range of drug concentrations as described. Chromatography, which was relatively time-consuming, could be fully automated with no need for technical supervision, since samples were stable for 24 h in the auto-sampler (data not shown). We utilized an ultraviolet detector, commonly used in hospital laboratories, but not expensive and sophisticated mass spectrometry. For the sample pretreatment, we chose liquid–liquid extraction, which is economical compared with solid-phase extraction. To simplify the experimental procedure, an internal standard was not used; nevertheless the method provided satisfactory validation results. Thus, this method would be suitable for routine TDM in conventional hospital laboratory settings.

Moreover, from a clinical point of view, this method is noteworthy for two reasons. First, this method covers most of key drugs currently used other than NRTIs. Since the standard treatment of HIV infection, HAART, consists of various combinations of anti-HIV drugs (Richman, 2001; British HIV Association Writing Committee, 2003; The Panel on Clinical Practices for Treatment of HIV Infection, 2004; Yeni *et al.*, 2004), it would be convenient to use TDM to measure plasma concentrations of several drugs simultaneously with a single method rather than to use each different method for each drug. Although NRTI concentrations cannot be determined with this method, NRTIs are pro-drugs that are converted to their active triphosphate forms within cells, and therefore TDM with plasma is considered less beneficial compared with PIs and NNRTIs,

which directly exert their antiviral effects (Back *et al.*, 2002). Secondly, this method allows the determination of the M8 metabolite of NFV as well as the parent NFV. NFV M8, which is produced by CYP 2C19 in the liver, is equipotent to the unchanged parent NFV against HIV *in vitro* (Zhang *et al.*, 2001), although almost all the metabolites of PIs and EFV have no obvious antiviral activity. The pharmacokinetics of NFV M8 would be also markedly affected by genetic background, drug interactions and hepatic dysfunction, leading to a wide inter-patient variability (Khaliq *et al.*, 2000). These findings suggest that measurement of the parent NFV plus its M8 metabolite in plasma would be preferable to that of the parent alone for TDM in NFV treatment.

TDM with this method is expected to contribute to the optimization of HIV treatment for individual patients through modification of dosage and assessment of adherence to treatment. This method would be also useful for studying the relationships between drug concentrations and efficacy or toxicity and for analyzing pharmacokinetics and drug interactions in heavily co-medicated patients. Such concentration-oriented approaches and studies are in progress in our hospital.

CONCLUSION

A simple, economical and reliable chromatographic method has been developed for the simultaneous determination of the six PIs, NFV M8 and EFV in plasma. This method would be useful for routine TDM and pharmacokinetic studies in patients receiving PIs and EFV.

Acknowledgments

The authors thank Hiroyuki Fukuda of Applied Biosystems Japan Ltd for helpful suggestions. The authors also thank Abbott Laboratories, Japan Tobacco Inc., Kissei Pharmaceutical Co., Merck & Co. and Roche Products for providing the reference compounds. This study was supported in part by Grants-in-Aid for AIDS research from the Ministry of Health, Labour and Welfare of Japan (H15-15100101), by the Japanese Foundation for AIDS Prevention, and by the Organization of Pharmaceutical Safety and Research (01-4).

REFERENCES

Acosta EP, Henry K, Baken L, Page LM and Fletcher CV. Indinavir concentrations and antiviral effect. *Pharmacotherapy* 1999; **19**: 708.

Acosta EP, Gerber JG and The adult pharmacology committee of the AIDS clinical trials group. Position paper on therapeutic drug monitoring of antiretroviral agents. *AIDS Research and Human Retroviruses* 2002; **18**: 825.

Back D, Gatti G, Fletcher C, Garaffo R, Haubrich R, Hoetelmans R, Kurowski M, Luber A, Merry C and Perno CF. Therapeutic drug monitoring in HIV infection: current status and future directions. *AIDS* 2002; **16**: S5.

Barry MG, Merry C, Lloyd J, Halifax K, Carey P, Mulcahy F and Back DJ. Variability in trough plasma saquinavir concentrations in HIV patients: a case for therapeutic drug monitoring. *British Journal of Clinical Pharmacology* 1998; **45**: 501.

British HIV Association Writing Committee. BHIVA guidelines for the treatment of HIV-infected adults with antiretroviral therapy: July 2003; www.bhiva.org (access date: 18 October 2004).

Crommentuyn KML, Rosing H, Nan-Offeringa LGAH, Hillebrand MJX, Huitema ADR and Beijnen JH. Rapid quantification of HIV protease inhibitors in human plasma by high-performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry. *Journal of Mass Spectrometry* 2003; **38**: 157.

Dresser GK, Spence JD and Bailey DG. Pharmacokinetic–pharmacodynamic consequences and clinical relevance of cytochrome P450 3A4 inhibition. *Clinical Pharmacokinetics* 2000; **38**: 41.

Fellay J, Marzolini C, Meaden ER, Back DJ, Buclin T, Chave JP, Decosterd LA, Furrer H, Opravil M, Pantaleo G, Retelska D, Ruiz L, Schinkel AH, Vernazza P, Eap CB and Telenti A. Response to antiretroviral treatment in HIV-1-infected individuals with allelic variants of the multidrug resistance transporter 1: a pharmacogenetics study. *Lancet* 2002; **359**: 30.

Frerichs VA, DiFrancesco R and Morse GD. Determination of protease inhibitors using liquid chromatography-tandem mass spectrometry. *Journal of Chromatography B* 2003; **787**: 393.

Gerber JG. Using pharmacokinetics to optimize antiretroviral drug-drug interactions in the treatment of human immunodeficiency virus infection. *Clinical Infectious Diseases* 2000; **30**: S123.

Hugen PW, Langebeek N, Burger DM, Zomer B, van Leusen R, Schuurman R, Koopmans PP and Hekster YA. Assessment of adherence to HIV protease inhibitors: comparison and combination of various methods, including MEMS (electronic monitoring), patient and nurse report, and therapeutic drug monitoring. *Journal of AIDS* 2002; **30**: 324.

Khaliq Y, Gallicano K, Seguin I, Fyke K, Carignan G, Bulman D, Badley A and Cameron DW. Single and multiple dose pharmacokinetics of nelfinavir and CYP2C19 activity in human immunodeficiency virus-infected patients with chronic liver disease. *British Journal of Clinical Pharmacology* 2000; **50**: 108.

Khoo SH, Gibbons SE and Back DJ. Therapeutic drug monitoring as a tool in treating HIV infection. *AIDS* 2001; **15**: S171.

Leibenguth P, Le Guellec C, Besnier JM, Bastides F, Mace M, Gaudet ML, Autret-Leca E and Paintaud G. Therapeutic drug monitoring of HIV protease inhibitors using high-performance liquid chromatography with ultraviolet or photodiode array detection. *Therapeutic Drug Monitoring* 2001; **23**: 679.

Marzolini C, Telenti A, Decosterd LA, Greub G, Biollaz J and Buclin T. Efavirenz plasma levels can predict treatment failure and central nervous system side effects in HIV-1-infected patients. *AIDS* 2001; **15**: 71.

Palella FJ Jr, Delaney KM, Moorman AC, Loveless MO, Fuhrer J, Satten GA, Aschman DJ and Holmberg SD. Declining morbidity and mortality among patients with advanced human immunodeficiency virus infection. *New England Journal of Medicine* 1998; **338**: 853.

Poirier JM, Robidou P and Jaillon P. Simultaneous determination of the six HIV protease inhibitors (amprenavir, indinavir, lopinavir, nelfinavir, ritonavir, and saquinavir) plus M8 nelfinavir metabolite and the nonnucleoside reverse transcription inhibitor efavirenz in human plasma by solid-phase extraction and column liquid chromatography. *Therapeutic Drug Monitoring* 2002; **24**: 302.

Rentsch KM. Sensitive and specific determination of eight antiretroviral agents in plasma by high-performance liquid chromatography-mass spectrometry. *Journal of Chromatography B* 2003; **788**: 339.

Rezk NL, Tidwell RR and Kashuba AD. High-performance liquid chromatography assay for the quantification of HIV protease inhibitors and non-nucleoside reverse transcriptase inhibitors in human plasma. *Journal of Chromatography B* 2004; **805**: 241.

Richman DD. HIV chemotherapy. *Nature* 2001; **410**: 995.

The Panel on Clinical Practices for Treatment of HIV Infection. Guidelines for the use of antiretroviral agents in HIV-1-infected adults and adolescents: March 23, 2004. AIDSinfo.nih.gov (access date: 18 October 2004).

Titier K, Lagrange F, Pehourcq F, Edno-Mcheik L, Moore N and Molimard M. High-performance liquid chromatographic method for the simultaneous determination of the six HIV-protease inhibitors and two non-nucleoside reverse transcriptase inhibitors in human plasma. *Therapeutic Drug Monitoring* 2002; **24**: 417.

Tribut O, Arvieux C, Michelet C, Chapplain JM, Allain H and Bentue-Ferrer D. Simultaneous quantitative assay of six HIV protease inhibitors, one metabolite, and two non-nucleoside reverse transcriptase inhibitors in human plasma by isocratic reversed-phase liquid chromatography. *Therapeutic Drug Monitoring* 2002; **24**: 554.

Turner ML, Reed-Walker K, King JR and Acosta EP. Simultaneous determination of nine antiretroviral compounds in human plasma using liquid chromatography. *Journal of Chromatography B* 2003; **784**: 331.

Van Heeswijk RP. Critical issues in therapeutic drug monitoring of antiretroviral drugs. *Therapeutic Drug Monitoring* 2002; **24**: 323.

Volosov A, Alexander C, Ting L and Soldin SJ. Simple rapid method for quantification of antiretrovirals by liquid chromatography–tandem mass spectrometry. *Clin. Biochem.* 2002; **35**: 99.

Yeni PG, Hammer SM, Hirsch MS, Saag MS, Schechter M, Carpenter CC, Fischl MA, Gatell JM, Gazzard BG, Jacobsen DM, Katzenstein DA, Montaner JS, Richman DD, Schooley RT, Thompson MA, Vella S and Volberding PA. Treatment for adult HIV infection: 2004 recommendations of the International AIDS Society—USA panel. *Journal of the American Medical Association* 2004; **292**: 251.

Zhang KE, Wu E, Patick AK, Kerr B, Zorbas M, Lankford A, Kobayashi T, Maeda Y, Shetty B and Webber S. Circulating metabolites of the human immunodeficiency virus protease inhibitor nelfinavir in humans: structural identification, levels in plasma, and antiviral activities. *Antimicrobial Agents and Chemotherapy* 2001; **45**: 1086.

Original Article

Difference of Progression to AIDS According to CD4 Cell Count, Plasma HIV RNA Level and the Use of Antiretroviral Therapy among HIV Patients Infected through Blood Products in Japan

Miyuki Kawado,[1] Shuji Hashimoto,[1] Takuhiro Yamaguchi,[2] Shin-ichi Oka,[3] Kazuyuki Yoshizaki,[4] Satoshi Kimura,[3] Katsuyuki Fukutake,[5] Satoshi Higasa,[6] and Takuma Shirasaka.[7]

BACKGROUND: It is important to examine progression to acquired immunodeficiency syndrome (AIDS) or death and its predictors among human immunodeficiency virus (HIV) infected persons before and after the introduction of the highly active antiretroviral therapy (HAART) available in Japan since 1997.

METHODS: The data used were from a survey of persons with HIV infected through blood coagulation factor products in Japan. Progression to AIDS or death during two periods, between January 1994 and March 1997, and between April 1997 and March 2002, were observed.

RESULTS: The AIDS-free proportion after 3 years was 74% among 417 participants for the earlier period and 94% among 605 participants in the later one. The hazard ratio of low CD4 cell count (less than 200 cells/ μL) was 50.8 for the earlier period and 4.7 for the later one compared with that of 500 cells/ μL or more. After adjustment by plasma HIV RNA levels and use of antiretroviral therapy, the hazard ratios of the low CD4 cell count for the later period were still significant.

CONCLUSION: The AIDS-free proportion among people with HIV infected through blood products in Japan largely increased after the introduction of HAART. The CD4 cell count remains an important predictor of future progression, but its importance might be less because of HAART.

J Epidemiol 2006; 16:101-106.

Key words: HIV; Acquired Immunodeficiency Syndrome; Blood Coagulation Factors; CD4 Lymphocyte Count; Antiretroviral Therapy, Highly Active.

It is a great challenge for both the public health and medical fields to discover the potential human immunodeficiency virus (HIV) infections[1] and to prevent HIV-infected persons from progressing to acquired immunodeficiency syndrome (AIDS) or death. In recent years, highly active antiretroviral therapy (HAART) including combination regimens such as two nucleoside reverse transcriptase inhibitors plus one protease inhibitor have become available.[2] It is well-known that they have had a significant impact on preventing or delaying AIDS progression for individuals with HIV,[3-5] whereas their actual impact on the entire HIV-

Received May 23, 2005, and accepted November 25, 2005.
This study was supported by the Yu-ai Welfare Foundation in Japan, and in part by the Pharmaceuticals and Medical Devices Agency of Japan, which provided the data.

[1] Department of Hygiene, Fujita Health University School of Medicine.
[2] Department of Biostatistics / Epidemiology and Preventive Health Sciences, School of Health Science and Nursing, University of Tokyo.
[3] AIDS Clinical Center, International Medical Center of Japan.
[4] Department of Medical Science I, School of Health and Sport Sciences, Osaka University.
[5] Department of Laboratory Medicine, Tokyo Medical University Hospital.
[6] Department of Internal Medicine, Hyogo College of Medicine.
[7] Osaka National Hospital.
Address for correspondence: Miyuki Kawado, Department of Hygiene, Fujita Health University School of Medicine, 1-98 Dengakugakubo, Kutsukake-cho, Toyoake City, Aichi 470-1192, Japan. (kawado@fujita-hu.ac.jp)

infected population has not been sufficiently evaluated except several European countries and the United States. Also in Japan, there is no precedent in terms of observational study.

The CD4 cell count and plasma HIV-RNA level were important for predicting future progression to AIDS or death among HIV-infected persons before the introduction of HAART,[6-8] but their implications may have been reduced since then.[9-11] There are a few reports on the predictors of AIDS progression before and after HAART became widely available.[9,11]

In the present study, we examined the progression to AIDS or death before and after the widespread use of HAART among people with HIV infected through blood products in Japan. The effect of the CD4 cell count as a predictor of progression to AIDS or death before and after HAART usage was evaluated. The effects of plasma HIV RNA level and use of antiretroviral therapy after HAART became available were also examined.

METHODS

Survey and Research Program for People with HIV Infected through Blood Products in Japan

In Japan, a survey and research program for people with HIV infection through the use of contaminated blood coagulation factor products has been carried out since 1993 fiscal year with the support of the Ministry of Health and Welfare.[12-14] This program is intended to help prevent them from developing HIV-infected symptoms in daily living by providing health management expenses. For this research, subjects were requested to submit reports filled out by their treatment physician on a quarterly basis, which included CD4 cell count and administered antiretroviral drugs. The plasma HIV RNA level was appended to this report from the second quarter of 1997. If subjects were diagnosed with AIDS,[15] they were excluded from this survey. The date of the diagnosis of AIDS or death was ascertained in the survey. Details of the survey had been described elsewhere.[12-14]

Data Analysis

The data from the survey mentioned above were made available, including sex, age, CD4 cell count, plasma HIV RNA level, antiretroviral therapy status, and the date of the diagnosis of AIDS onset or death. No personal identifiers such as name or address were included. We drew two subset cohorts from that data for our analysis. One cohort consisted of participants on January 1, 1994. The other consisted of participants as of April 1, 1997 because HAART became available in Japan in 1997. The first subset cohort was used in the analysis of progression to AIDS or death before 1997, and the second subset cohort was used in that after 1997.

For the analysis of progression to AIDS or death before 1997, the data of progression to AIDS or death between January 1, 1994 and March 31, 1997 were used. AIDS-free proportion by CD4 cell count in the first quarter of 1994 was estimated using Kaplan-Meier methods. The Cox proportional hazards model was used to estimate the hazard ratio of the CD4 cell count for progression to AIDS or death and its 95% confidence interval. Because the data did not include plasma HIV RNA levels, and use of antiretroviral therapy was rare in the first quarter of 1994, we did not use these variables in this analysis.

For the analysis of progression to AIDS or death after 1997, the data between April 1, 1997 and March 31, 2002 were used. AIDS-free proportions, hazard ratios for progression to AIDS or death and their 95% confidence intervals were estimated by CD4 cell count, plasma HIV RNA level and the use of antiretroviral therapy in the second quarter of 1997. Hazard ratios by combinations of these three variables were also estimated.

The CD4 cell count was divided into four categories: less than 200, 200-349, 350-499, and 500 cells/μL or more. The plasma HIV RNA level was divided into six categories: less than 400, 400-999, 1,000-4,999, 5,000-9,999, 10,000-49,999 and 50,000 copies/mL or more. Use of antiretroviral therapy was classified into five categories: given no treatment (No treatment), treatments including only one nucleoside reverse transcriptase inhibitor (1 NRTI), those including only two NRTIs (2 NRTIs), those including at least two NRTIs and one protease inhibitor (2 NRTIs + 1 PI), and other treatments (Other treatments). For estimating hazard ratios by combinations of the three variables, we divided each variable into two categories before putting them together; CD4 cell count (less than 200 vs. 200 cells/μL or more), plasma HIV RNA level (less than 50,000 vs. 50,000 copies/mL or more), and the use of antiretroviral therapy (2 NRTIs + 1 PI vs. not 2 NRTIs + 1 PI).

All analyses were conducted using SAS® software, version 8.2 (SAS Institute, Inc., Cary, NC, USA).[16]

RESULTS

Analysis of Progression to AIDS or Death before 1997

The number of participants as of January 1, 1994 was 417 (415 males and 2 females). Mean age was 27.9 (standard deviation = 10.7) years. Of these, 113 participants progressed to AIDS or death, and no participant was lost to follow-up by March 31, 1997. The proportion of AIDS-free participants after 3 years was 0.74.

Figure 1 shows AIDS-free proportions before April 1997 by CD4 cell counts in the first quarter of 1994. Among 400 participants whose CD4 cell count was available, the AIDS-free proportion decreased rapidly each year where the CD4 cell count was less than 200 cells/μL.

As shown in Table 1, the hazard ratio of progression to AIDS or death before April 1997 was 50.8 ($p<0.01$) in CD4 cell counts of less than 200 cells/μL and 7.7 ($p=0.05$) in those of 200-349 cells/μL compared with those of 500 cells/μL or more.

Analysis of Progression to AIDS or Death after 1997

The number of participants at April 1, 1997 was 605 (583 males and 22 females). Mean age was 29.7 (standard deviation = 10.1)

Table 1. Hazard ratios of CD4 cell count in the first quarter of 1994 for progression to AIDS or death before 1997.

CD4 cell count (cells / μ L)	n	Hazard ratio	95% confidence interval	p-value
<200	125	50.79	7.06 - 365.30	<0.01
200 – 349	118	7.73	1.03 - 58.25	0.05
350 – 499	96	2.81	0.33 - 24.01	0.35
≥500	53	1	(reference)	

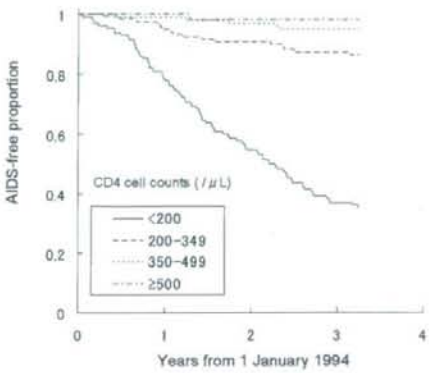

Figure 1. AIDS-free proportion between January 1, 1994 and March 31, 1997 by CD4 cell count in the first quarter of 1994.

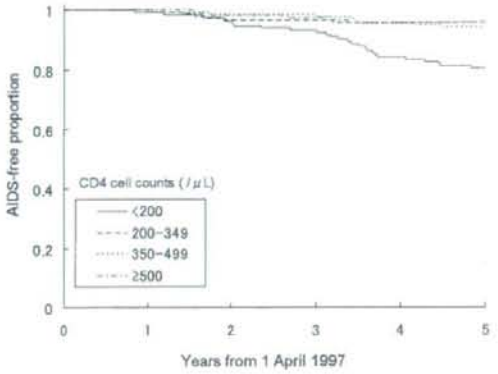

Figure 2. AIDS-free proportion between April 1, 1997 and March 31, 2002 by CD4 cell count in the second quarter of 1997.

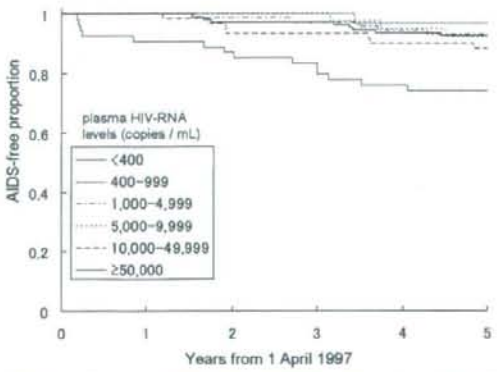

Figure 3. AIDS-free proportion between April 1, 1997 and March 31, 2002 by plasma HIV RNA level in the second quarter of 1997.

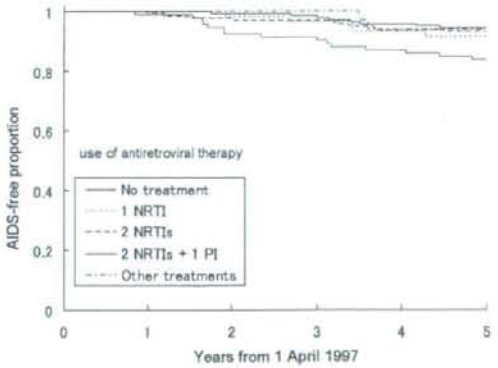

Figure 4. AIDS-free proportion between April 1, 1997 and March 31, 2002 by the use of antiretroviral therapy in the second quarter of 1997.

* 1 NRTI: treatments including only one nucleoside reverse transcriptase inhibitor.
2 NRTIs: treatments including only two nucleoside reverse transcriptase inhibitors.
2 NRTIs + 1 PI: treatments including at least two nucleoside reverse transcriptase inhibitors and one protease inhibitor.

Table 2. Hazard ratios of CD4 cell count, plasma HIV RNA level and the use of antiretroviral therapy in the second quarter of 1997 for progression to AIDS or death after 1997.

variable	n	Hazard ratio	95% confidence interval	p-value
CD4 cell count (cells / μL)				
<200	133	4.74	1.82 - 12.35	<0.01
200 – 349	144	0.96	0.29 - 3.13	0.94
350 – 499	134	1.36	0.45 - 4.16	0.59
≥500	114	1	(reference)	
Plasma HIV RNA level (copies / mL)				
<400	108	1	(reference)	
400 – 999	30	0.44	0.06 - 3.50	0.44
1,000 – 4,999	71	0.94	0.31 - 2.88	0.92
5,000 – 9,999	38	1.04	0.28 - 3.93	0.95
10,000 – 49,999	60	1.61	0.58 - 4.45	0.36
≥50,000	50	2.94	1.16 - 7.45	0.02
Use of antiretoroviral therapy*				
No treatment	144	1	(reference)	
1 NRTI	58	1.57	0.52 - 4.81	0.43
2 NRTIs	214	1.28	0.54 - 3.03	0.57
2 NRTIs + 1 PI	93	3.13	1.33 - 7.37	0.01
Other treatments	33	1.08	0.23 - 5.10	0.92

* 1 NRTI: treatments including only one nucleoside reverse transcriptase inhibitor.
2 NRTIs: treatments including only two nucleoside reverse transcriptase inhibitors.
2 NRTIs + 1 PI: treatments including at least two nucleoside reverse transcriptase inhibitors and one protease inhibitor.

Table 3. Hazard ratios of combination of CD4 cell count, plasma HIV RNA level and the use of antiretroviral therapy in the second quarter of 1997 for progression to AIDS or death after 1997.

CD4 cell counts (cells / μ L)	Plasma HIV RNA level (copies / mL)	Use of antiretroviral treatment*	n	Hazard ratio	95% confidence interval	p-value
≥200	<50,000	not 2NRTIs + 1 PI	216	1	(reference)	
≥200	<50,000	2 NRTIs + 1 PI	27	2.35	0.65 - 8.41	0.19
≥200	≥50,000	not 2NRTIs + 1 PI	19	1.04	0.13 - 8.01	0.97
≥200	≥50,000	2 NRTIs + 1 PI	4	0.00	0.00 - —	0.99
<200	<50,000	not 2NRTIs + 1 PI	41	2.89	1.07 - 7.81	0.04
<200	<50,000	2 NRTIs + 1 PI	23	3.51	1.12 - 11.02	0.03
<200	≥50,000	not 2NRTIs + 1 PI	13	5.02	1.40 - 18.01	0.01
<200	≥50,000	2 NRTIs + 1 PI	13	12.42	4.59 - 33.65	<0.01

* 2 NRTIs + 1 PI: treatments including at least two nucleoside reverse transcriptase inhibitors and one protease inhibitor.

years. Of these, 65 participants progressed to AIDS or death and 1 participant was lost to follow-up by March 31, 2002. The proportion of AIDS-free participants was 0.94 after 3 years and 0.89 after 5 years.

Figures 2, 3 and 4 show the AIDS-free proportion between April 1997 and March 2002 by CD4 cell count, plasma HIV RNA level, and the use of antiretroviral therapy at the second quarter of 1997, respectively. The number of participants in this analysis was 525 for CD4 cell count, 357 for plasma HIV RNA level and 542 for use of antiretroviral therapy because of missing data. The AIDS-free proportion was lower where the CD4 cell count was less than 200 cells/ μL, with plasma HIV RNA levels of 50,000 copies/mL or more and also lower in 2 NRTIs + 1 PI than in the others.

Table 2 shows the hazard ratios of CD4 cell count, plasma HIV RNA level and use of antiretroviral therapy at the second quarter of 1997 for progression to AIDS or death between April 1997 and March 2002. The hazard ratio was 4.7 ($p<0.01$) in a CD4 cell count of less than 200 cells/ μL compared with those of 500 cells/ μL or more, 2.9 ($p=0.02$) in those of 50,000 copies/mL or more compared with those with less than 400 copies/mL, and 3.1 ($p=0.01$) in 2 NRTIs + 1 PI compared with no treatment.

Table 3 shows the hazard ratios of the combinations of CD4 cell count, plasma HIV RNA level, and use of antiretroviral therapy in the second quarter of 1997 for progression to AIDS or death between April 1997 and March 2002. The hazard ratio was significantly higher in each of the combinations with a CD4 cell count of less than 200 cells/ μL than in the combination with CD4 cell counts of 200 cells/ μL or more, plasma HIV RNA levels of less than 50,000 copies/mL, and not 2 NRTIs + 1 PI.

DISCUSSION

Among people with HIV infected through blood coagulation factor products, the AIDS-free proportion before 1997 was markedly lower than that after 1997, when HAART was widely used in Japan. This would reflect the strong effect of HAART for preventing or delaying the progression to AIDS or death. Several studies conducted in Europe or the United States indicated results similar to those of the present study.[9,11]

The hazard ratio for progression to AIDS or death before 1997 was 50.8 in CD4 cell counts of less than 200 cells/ μL compared with those of 500 cells/ μL or more. This result was consistent with the previous reports,[9,11] showing that a low CD4 cell count was an important predictor of future progression to AIDS or death among HIV-infected persons before the introduction of HAART. The hazard ratio of a CD4 cell count of less than 200 cells/ μL for progression to AIDS or death after 1997 was 4.7. These results suggested that while the CD4 cell count was still an important predictor of future progression to AIDS or death after the introduction of HAART, its importance has lessened because of the introduction of HAART.[17]

The AIDS-free proportion after 1997 was lower in plasma HIV RNA levels of 50,000 copies/mL or more than in other levels. The hazard ratio of the combination of higher plasma HIV RNA level and lower CD4 cell count was significant, whereas that of the combination of higher plasma HIV RNA level and higher CD4 cell count was not. Thus, the plasma HIV RNA level, as a predictor of future progression to AIDS or death, might be less important than the CD4 cell count. This suggestion would be consistent with the recommendations for treatments of HIV-infected persons: the CD4 cell count could be used as a marker for beginning HAART, and the plasma HIV RNA level may be used as a marker for monitoring the effect of HAART treatments.[18,19]

We observed that the AIDS-free proportion after 1997 was lower in 2 NRTIs + 1 PI than in the others. The hazard ratio of the combination of 2 NRTI + 1 PI and lower CD4 cell count was significant, but that of the combination of 2 NRTIs + 1 PI and higher CD4 cell count was not. These results do not indicate the ineffectiveness of the treatment with 2 NRTIs + 1 PI; rather, it would reflect that patients treated by 2 NRTIs + 1 PI had a higher risk of progression to AIDS or death if their CD4 cell counts after the use of HAART were still low.[3]

The present analysis has several problems and limitations. We used data from a survey of patients with HIV infection through blood coagulation factor products in Japan. All people infected with HIV through blood coagulation factor products were not subjects of this survey because they needed to make an application for participation and then had to be approved as subjects. The coverage of the subjects in April 1997 was about 80%. We extracted two subset cohorts from the data for our analysis. Therefore, the later cohort contains the former cohort's subject who survived and did not develop AIDS until March 31, 1997. The results from one cohort might not be strictly comparable with those from the other cohort. The data on the progression to AIDS or death were ascertained in the survey, and were used as evidence for disease progression; however, some of the deaths could have been caused by other diseases without having progressed to AIDS. The data we used did not include the cause of death. In our analysis of the progression to AIDS or death, other variables such as sex and age were not included. The date of the diagnosis of HIV infection was also not included because data were not available. We focused on the effects of variables as a predictor for future progression to AIDS or death. Future progression to AIDS or death could be strongly influenced by future treatments. Most of our subjects would have received adequate treatments during the period when the progression to AIDS or death was observed.

Although our findings might be restricted by some of the problems and conditions mentioned above, this study should help to evaluate the actual impact of the widespread use of HAART on the HIV-infected population in non-Western countries, and to provide information on the importance of CD4 cell counts and plasma HIV RNA levels as predictors of future progression to AIDS or death.

REFERENCES

1. Weinstock H, Dale M, Linley L, Gwinn M. Unrecognized HIV infection among patients attending sexually transmitted disease clinics. Am J Public Health 2002; 92: 280-3.
2. Hammer SM, Squires KE, Hughes MD, Grimes JM, Demeter LM, Currier JS, et al. A controlled trial of two nucleoside analogues plus indinavir in persons with human immunodeficiency virus infection and CD4 cell counts of 200 per cubic millimeter or less. N Engl J Med 1997; 337: 725-33.
3. Jacobson LP, Li R, Phair J, Margolick JB, Rinaldo CR, Detels R, et al. Evaluation of the effectiveness of highly active antiretroviral therapy in persons with human immunodeficiency virus using biomarker-based equivalence of disease progression. Am J Epidemiol 2002; 155: 760-70.
4. van Sighem AI, van de Wiel MA, Ghani AC, Jambroes M, Reiss P, Gyssens IC, et al. Mortality and progression to AIDS after starting highly active antiretroviral therapy. AIDS 2003; 17: 2227-36.
5. Cole SR, Hernan MA, Robins JM, Anastos K, Chmiel J, Detels R, et al. Effect of highly active antiretroviral therapy on time to acquired immunodeficiency syndrome or death using marginal structural models. Am J Epidemiol 2003; 158: 687-94.
6. Phillips AN, Sabin CA, Elford J, Bofill M, Janossy G, Lee CA. Use of CD4 lymphocyte count to predict long-term survival free of AIDS after HIV infection. BMJ 1994; 309: 309-13.
7. Mellors JW, Rinaldo CR Jr, Gupta P, White RM, Todd JA, Kingsley LA. Prognosis in HIV-1 infection predicted by the quantity of virus in plasma. Science 1996; 272: 1167-70.
8. Begtrup K, Melbye M, Biggar RJ, Goedert JJ, Knudsen K, Andersen PK. Progression to acquired immunodeficiency syndrome is influenced by CD4 T-lymphocyte count and time since seroconversion. Am J Epidemiol 1997; 145: 629-35.
9. Detels R, Munoz A, McFarlane G, Kingsley LA, Margolick JB, Giorgi J, et al. Effectiveness of potent antiretroviral therapy on time to AIDS and death in men with known HIV infection duration. Multicenter AIDS Cohort Study Investigations. JAMA 1998; 280: 1497-503.
10. Hogg RS, Yip B, Chan KJ, Wood E, Craib KJ, O'Shaughnessy MV, et al. Rates of disease progression by baseline CD4 cell count and viral load after initiating triple-drug therapy. JAMA 2001; 286: 2568-77.
11. Mocroft A, Ledergerber B, Katlama C, Kirk O, Reiss P, d'Arminio Monforte A, et al. Decline in the AIDS and death rates in the EuroSIDA study: an observational study. Lancet 2003; 362: 22-9.
12. Hashimoto S, Yamaguchi T, Oka S, Yoshizaki K, Kimura S, Fukutake K, et al. CD4 cell counts, plasma HIV-RNA levels and use of antiretroviral therapy among patients with HIV infection through blood products. J AIDS Res 2001; 3: 16-22. (in Japanese)
13. Yamaguchi T, Hashimoto S, Oka S, Yoshizaki K, Kimura S, Fukutake K, et al. Trends in CD4 cell counts, plasma HIV-RNA levels and the use of antiretroviral therapy, and those relationships among patients with HIV infection through blood products. J AIDS Res 2001; 3: 23-30. (in Japanese)
14. Yamaguchi T, Hashimoto S, Oka S, Yoshizaki K, Kimura S, Fukutake K, et al. Physical condition and activity of daily living among HIV patients infected through blood products in Japan. J Epidemiol 2002; 12: 383-93.
15. Guideline of an infectious disease report —a standard of an infectious disease diagnosis —. Association of Japan public sanitation Foundation, 2005 (in Japanese).
16. SAS Institute Inc. SAS/STAT User's Guide, version 8. SAS Institute Inc. Cary, NC, 1999.
17. Dybul M, Fauci AS, Bartlett JG, Kaplan JE, Pau AK; Panel on Clinical Practices for the Treatment of HIV. Guidelines for using antiretroviral agents among HIV-infected adults and adolescents. Recommendations of the Panel on Clinical Practices for Treatment of HIV. MMWR Recomm Rep 2002; 51(RR-7): 1-55.
18. Cozzi Lepri A, Phillips AN, d'Arminio Monforte A, Castelli F, Antinori A, de Luca A, et al. When to start highly active antiretroviral therapy in chronically HIV-infected patients: evidence from the ICONA study. AIDS 2001; 15: 983-90.
19. Ahdieh-Grant L, Yamashita TE, Phair JP, Detels R, Wolinsky SM, Margolick JB, et al. When to initiate highly active antiretroviral therapy: a cohort approach. Am J Epidemiol 2003; 157: 738-46.

Available online at www.sciencedirect.com
SCIENCE DIRECT
Antiviral Research 70 (2006) 51–59
Antiviral Research
www.elsevier.com/locate/antiviral

Cooperative contribution of gag substitutions to nelfinavir-dependent enhancement of precursor cleavage and replication of human immunodeficiency virus type-1

Saori Matsuoka-Aizawa [a,b], Hiroyuki Gatanaga [a,*], Hironori Sato [c], Kazuhiko Koike [b], Satoshi Kimura [a], Shinichi Oka [a]

[a] *AIDS Clinical Center, International Medical Center of Japan, 1-21-1, Toyama, Shinjuku-ku, Tokyo 162-8655, Japan*
[b] *Graduate School of Medicine, University of Tokyo, Japan*
[c] *Division of Molecular Genetics, National Institute of Infectious Diseases, Tokyo, Japan*

Received 9 June 2005; accepted 11 January 2006

Abstract

We previously described a clinical human immunodeficiency virus type-1 (HIV-1) isolate, CL-4, which showed nelfinavir (NFV)-dependent enhancement of replication (Matsuoka-Aizawa, S., Sato, H., Hachiya, A., Tsuchiya, K., Takebe, Y., Gatanaga, H., Kimura, S., Oka, S, 2003. Isolation and molecular characterization of a nelfinavir (NFV)-resistant human immunodeficiency virus type 1 that exhibits NFV-dependent enhancement of replication. J. Virol. 77, 318–327.). To identify the responsible region(s) of HIV-1 proteins for such replication enhancement, we constructed a panel of recombinant HIV-1 clones harboring portions of the Gag and protease of CL-4 and analyzed their replication capabilities and Gag processing patterns. Our data suggested that the substitutions in the matrix and N-terminal half of capsid of CL-4 were indispensable for the NFV-dependent enhancement of replication and that NFV facilitated the cleavage between the matrix and capsid of the Gag precursor harboring these substitutions. The substitutions in C-terminal half of capsid rather decreased the cleavability of Gag precursor and NFV counteracted such negative impact. Efficient replication enhancement with NFV can be observed only in the presence of the substitutions in entire Gag and protease of CL-4.

Keywords: Human immunodeficiency virus type 1; Neltinavir-resistant; Gag mutation

1. Introduction

Under the selective pressure of antiretroviral agents, the human immunodeficiency virus type-1 (HIV-1) evolves and acquires drug-resistance-associated mutations. The major protease inhibitor (PI)-resistance-associated mutations are located in the active sites of HIV-1 protease and impair its enzymatic functions (Bleiber et al., 2001; Croteau et al., 1997; Martinez-Picado et al., 1999). In order to compensate such impaired enzymatic function, PI-resistant HIV-1 further acquires mutations not only in protease but also in one of its substrate, Gag, resulting in full recovery of replication ability (Doyon et al., 1996; Gatanaga et al., 2002; Tamiya et al., 2004; Zhang et al., 1997). We previously described a unique clinical HIV-1 isolate, CL-4, which replicated more efficiently in the presence of sub-inhibitory concentrations of nelfinavir (NFV) (0.001–0.1 μM) (Matsuoka-Aizawa et al., 2003). CL-4 had a total of 56 amino acid substitutions in *gag-pro* genes compared with NL4-3; 22 substitutions had emerged in the matrix, SP1, and protease during administration of NFV-containing therapy, and 34 other substitutions had already existed before the introduction of the therapy (Matsuoka-Aizawa et al., 2003). In that study, we constructed three HIV-1 clones including, p17PRmt, PRmt, and p24PRmt, and found that only p17PRmt, which possessed the entire Gag and protease segment of CL-4, showed NFV-dependent enhancement of replication. Therefore, we concluded that the substitutions in matrix are indispensable for replication enhancement (Matsuoka-Aizawa et al., 2003). However, it is still unknown whether the substitutions in matrix alone are sufficient or whether other Gag substitutions are necessary for the replication enhancement with NFV. In this study we constructed four more recombinant HIV-1 clones and characterized their replica-

* Corresponding author. Tel.: +81 3 5273 5193; fax: +81 3 5273 5193.
E-mail address: higatana@imcj.acc.go.jp (H. Gatanaga).

0166-3542/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.antiviral.2006.01.004

tion kinetics and Gag processing in the absence and presence of NFV.

2. Materials and methods

2.1. Cells and antiretroviral agents

HeLa cells were maintained in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal calf serum (FCS). Transformed T cell lines, MT-2, PM-1, and H9 cells were maintained in RPMI-1640 with 10% FCS. NFV was kindly provided by the Japan Tobacco Co. (Tokyo, Japan).

2.2. Plasmid construction and preparation of gag-pro recombinant HIV-1 clones

Clinical HIV-1 isolates CL-1, CL-2, CL-3, and CL-4 were sequentially obtained from the same patient before and during NFV-containing treatment (Matsuoka-Aizawa et al., 2003). Direct sequences of these four clinical isolates and sub-cloning

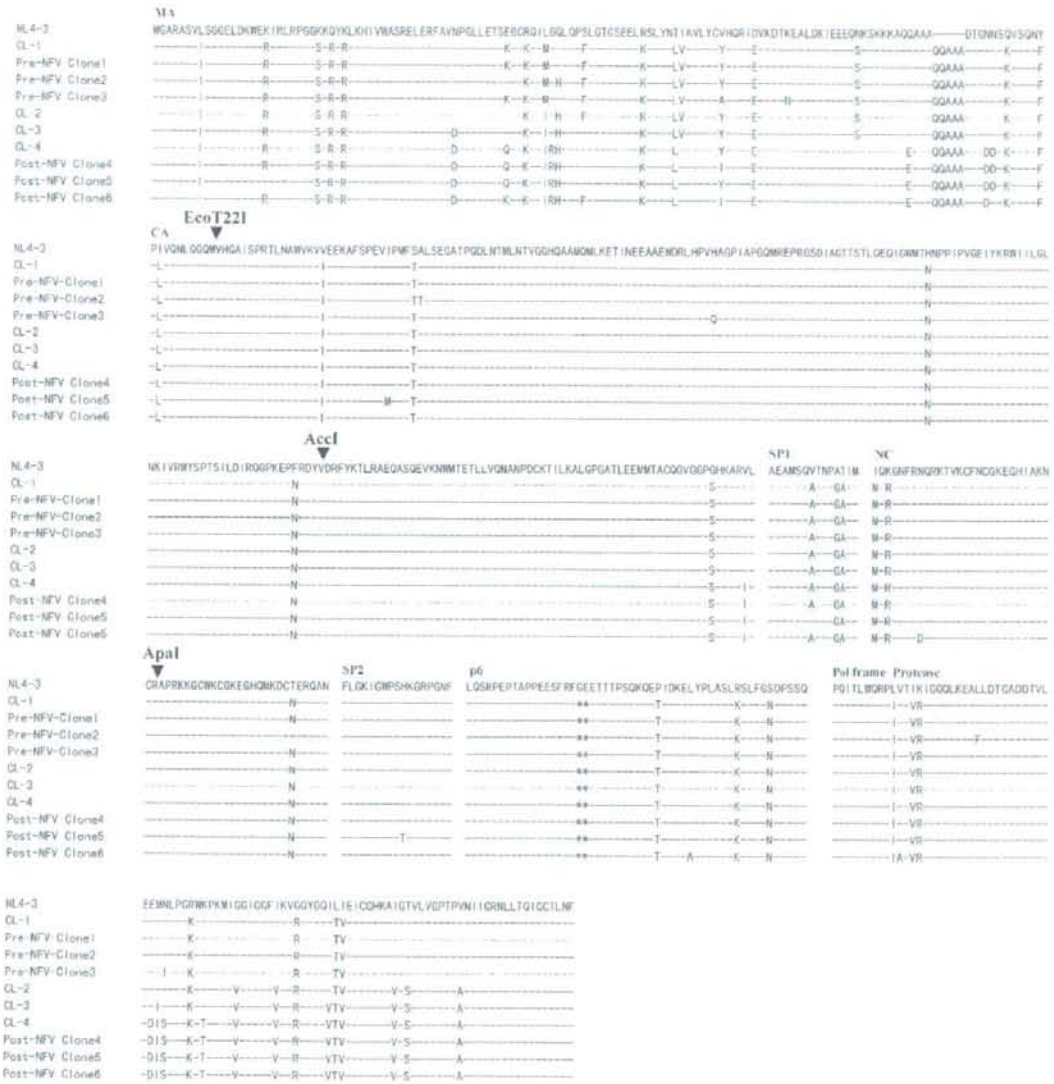

Fig. 1. Direct and sub-clonal sequences of clinical HIV-1 isolates. Direct and sub-clonal amino acid sequences of whole Gag and protease of HIV-1 isolates are shown. Pre-NFV Clone 1–3 and Post-NFV Clone 4–6 were derived from CL-1 and CL-4, respectively. The amino acid sequence of HIV-1_{NL4-3} is shown at the top as reference. The identical amino acids with those of HIV-1_{NL4-3} are indicated with dashes and the star shows deletion compared with HIV-1_{NL4-3} sequence. The restriction sites used in the construction of recombinant HIV-1 plasmids are also shown. MA, matrix; CA, Capsid; NC, nucleocapsid; and PR, protease.

sequences of CL-1 and CL-4 indicated that 11 and 10 amino acid substitutions accumulated in Gag and protease during PI-containing treatment, respectively (Fig. 1). Post-NFV Clone 4 (Fig. 1) was used in the construction of CL-4-derived recombinant HIV-1 plasmid. The pNL4-3-based plasmids of PRmt (HIV-1 carrying only the substitutions in protease of CL-4), p24PRmt (carrying the substitutions in capsid and protease of CL-4), and p17PRmt (carrying the substitutions in whole Gag and protease of CL-4) were constructed as previously described (Matsuoka-Aizawa et al., 2003) (Fig. 2), and the plasmids of MAmt (carrying only the substitutions in the matrix of CL-4) and MA + PRmt (carrying the substitutions in the matrix and protease of CL-4) were constructed by using the same restriction enzyme sites (Figs. 1 and 2). The plasmids of NCAmt (carrying the substitutions in matrix, N-terminal half of capsid, and protease of CL-4) and CCAmt (carrying the substitutions in matrix, C-terminal half of capsid, and protease of CL-4) were constructed by using AccI site. Originally, pNL4-3 has two AccI site between *gag* and protease region, one in the matrix, and the other in the capsid. However, since the one in the matrix was extinct due to natural substitution in CL-4, the other in the capsid was unique in *gag* and protease region.

HeLa cells (5×10^5 cells) were grown in DMEM with 10% FCS for 24 h and transfected with 3 μg of pNL4-3 and *gag*-protease recombinant HIV-1 plasmid DNAs by using FuGINE 6 transfection reagent (Roche Diagnosis, Basel, Switzerland). The cells were incubated for 24 h, washed once with PBS, and cultured in 5 ml of culture medium. The culture supernatant containing virus was collected at 48 h after transfection, filtered, analyzed for RT activity (10432–17162 cpm/μM), and kept at −80 °C until use. The virus titer used for infection and Western blot analysis was adjusted with RT activity.

2.3. HIV-1 replication kinetics

The methods used to infect cells were described previously (Matsuoka-Aizawa et al., 2003). Briefly, MT-2, PM-1, and H9 cells (2×10^4) were infected with 200 μl of cell-free supernatant containing HIV-1 (2×10^5 ^{32}P cpm of RT activity) in the absence or presence of NFV (0.1 and 1 μM) for 16 h, washed once, and cultured in 200 μl of culture medium with the same concentration of NFV. A half volume of culture medium was changed every 2 or 3 days, and the supernatant was kept at −80 °C for measurement of RT activity. Each experiment was carried out in duplicate and repeated three times.

2.4. Competitive HIV-1 replication assay

H9 cells (2×10^5 cells) were incubated with two HIV-1 clones (each of 100 $TCID_{50}$) simultaneously for 16 h, washed with PBS twice, and cultured in the absence or presence of 0.1 μM NFV for 7 days. These infection periods were defined as a single passage. At the end of each passage, H9 cells were harvested and the culture supernatants were used to infect fresh

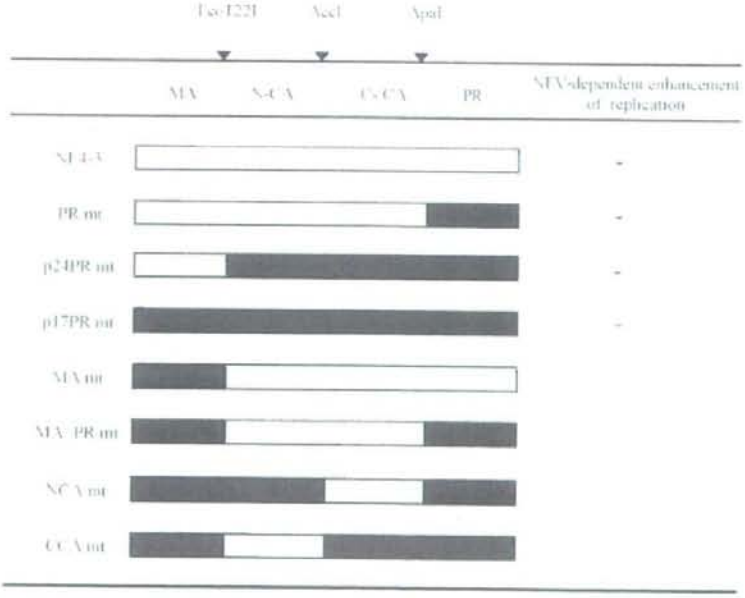

Fig. 2. Previously and newly constructed recombinant HIV-1s. The recombinant molecular clones were constructed based on pNL4-3 as a genetic backbone. The Gag-PR region of HIV-1 was segmented into four areas, MA (BssHII-EcoT22I fragment), N-terminal half of CA (NCA) (EcoT22I-AccI fragment), C-terminal half of CA (CCA) (AccI-ApaI fragment), and PR (ApaI-BalI fragment). Originally, pNL4-3 has two AccI sites between the gag and PR region, in MA and CA. However, because the one in MA was extinct in CL-4 due to natural substitution, the other AccI site in CA was unique for gag-PR gene of CL-4. Open boxes indicate the NL4-3-originated fragments, and closed boxes indicate fragments that were derived from CL-4 variants. The NFV-dependent replication enhancement of previously analyzed clones was also shown and indicated as (+). MA, matrix; CA, capsid; and PR, protease.