Competitive HIV-1 replication assay

Freshly prepared H9 cells (3×10^5) were exposed to the mixtures of paired virus preparations (300 BFU each) (HIV-1_{WT} vs. HIV-1_{K103R} in Figs. 4A–C, HIV-1_{WT} vs. HIV-1_{K103N} in Figs. 4D–F, and HIV-1_{WT} vs. HIV-$1_{K103R/V179D}$ in Figs. 4G–I) to be examined for their replication ability for 2 h, washed twice with PBS, and cultured in the absence (Figs. 4A, D, and G) or presence of EFV (3 nM for Fig. 4B, 10 nM for Figs. 4E and H) or NVP (30 nM for Fig. 4C, 100 nM for Figs. 4F and I) as describe previously (Hachiya et al., 2004; Kosalaraksa et al., 1999). On day 1, one-third of infected H9 cells were harvested and washed twice with PBS, and proviral DNAs were sequenced (0 week). Every 7 days, the supernatant of the virus culture was transmitted to new uninfected H9 cells, the cells harvested at the end of each passage (1, 2, and 3 weeks) were subjected to direct DNA sequencing of HIV-1 RT gene, and the viral population change was determined by the relative peak height on sequencing electrogram. The persistence of the original amino acid substitution was confirmed for all infectious clones used in this assay.

Acknowledgments

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

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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; Nelfinavir-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-

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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

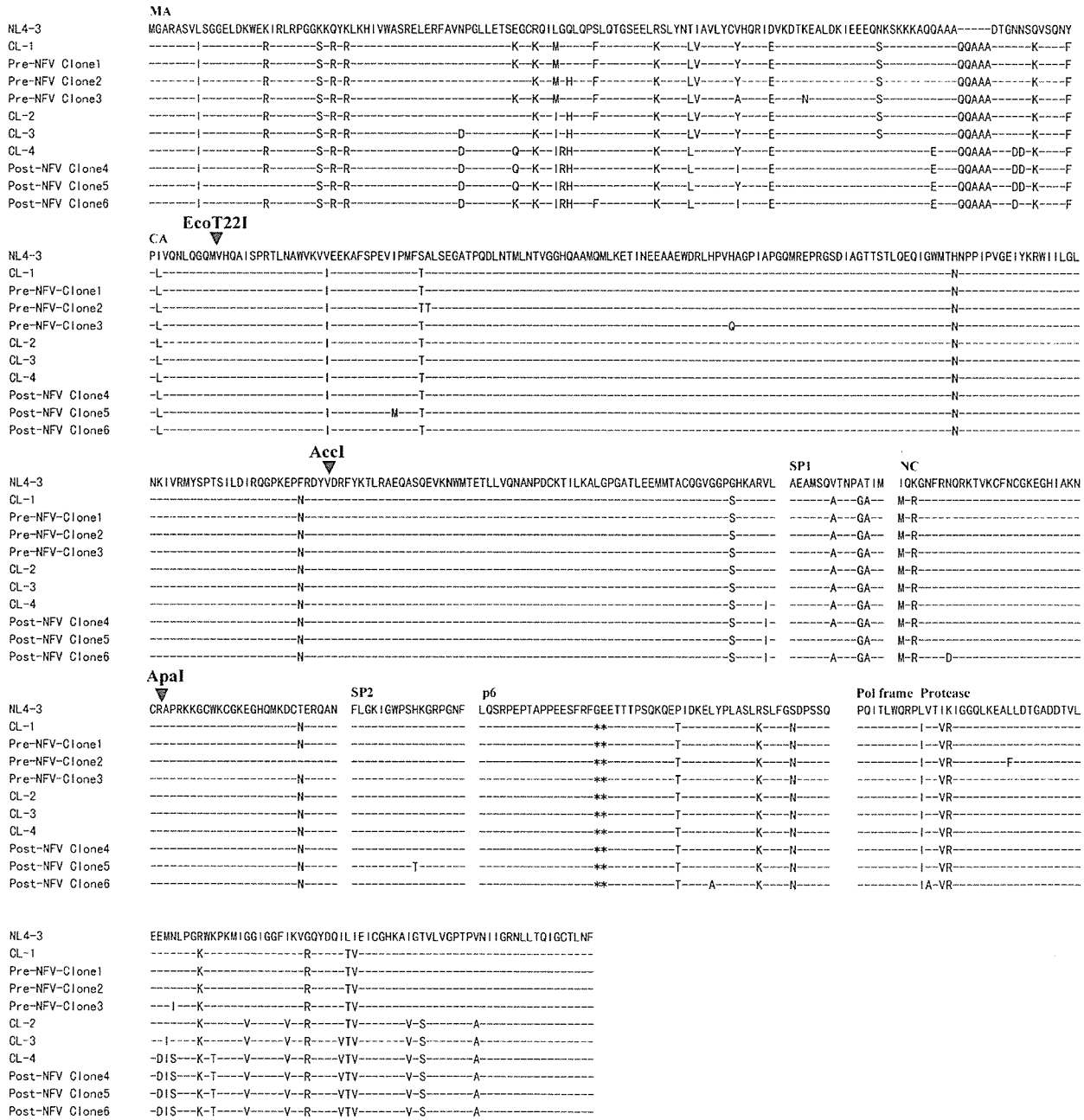

Fig. 1. Direct and sub-clonal sequences of clinical HIV-1 isolates. Direct and sub-clonal amino acid sequences of whole Gag and protease of HIV-1 isolates are shown. Pre-NFV Clone 1–3 and Post-NFV Clone 4–6 were derived from CL-1 and CL-4, respectively. The amino acid sequence of HIV-1_{NL4-3} is shown 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

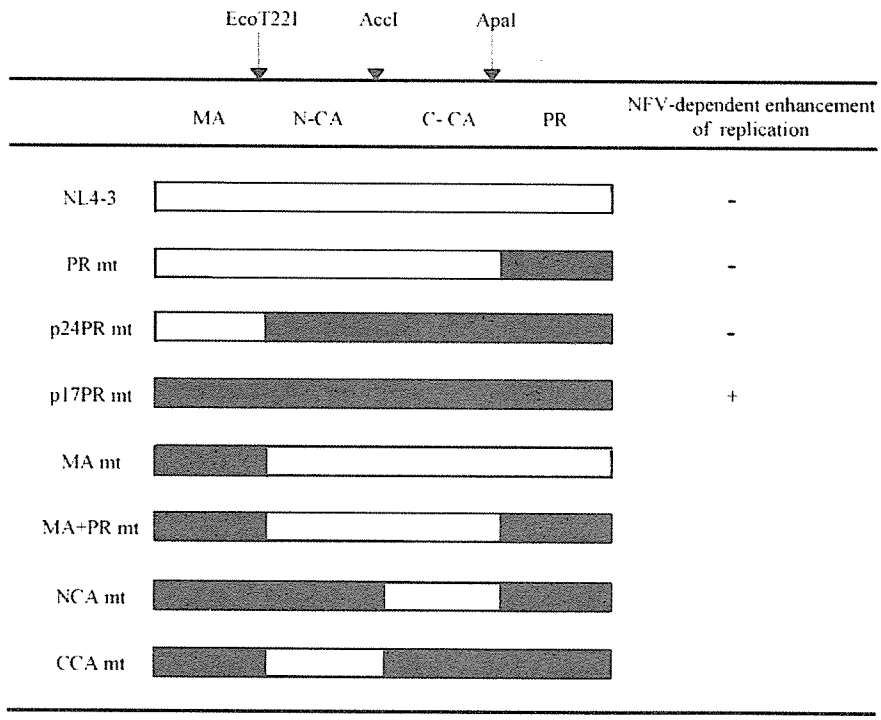

Fig. 2. Previously and newly constructed recombinant HIV-1s. The recombinant molecular clones were constructed based on pNL4-3 as a genetic backbone. The Gag-PR region of HIV-1 was segmented into four areas, MA (BssHII-EcoT22I fragment), N-terminal half of CA (NCA) (EcoT22I-AccI fragment), C-terminal 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.

uninfected H9 cells. The cells harvested at each passage were subjected to PCR for amplification of HIV-1 *gag* region and direct DNA sequencing was performed. The viral populational changes were determined by relative peak height on sequence electrophoregram (Kosalaraksa et al., 1999).

2.5. HIV-1 susceptibility to NFV

MT-2 cells were infected with 500 $TCID_{50}$ of each virus in the absence and the presence of 0.001, 0.00316, 0.01, 0.0316, 0.1, 0.316, 1, and 3.16 μM of NFV, and cultured in triplicate for 7 days. At the end of culture, the amounts of p24 in the supernatants were measured and 50% inhibitory concentrations (IC_{50}) of NFV were determined by referring to the dose–response curve.

2.6. Western blot analysis of HIV-1 virions

HeLa cells were transfected with pNL4-3 and *gag*-protease recombinant HIV-1 plasmid DNA in the absence and presence of 0.1 μM NFV. The culture supernatant was harvested at 48 h after transfection, centrifuged at 37,000 × *g* for 90 min to pellet virus particles. The virion pellet (6×10^5 cpm of RT activity) was applied to an SDS gradient gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was incubated with anti-HIV-1 p24 antisera (Advanced Biotechnology, Columbia, USA) and HIV-1-infected patients' serum, respectively, and hybridized with anti-protein A antibody conjugated with horseradish peroxidase (Amersham Pharmacia Biotech, Uppsala, Sweden). The immune complex was visualized with an ECL Plus system (Amersham Pharmacia Biotech) according to the manufactures' description.

The percent signal density of Gag products was analyzed on a Windows computer by using the ImageJ Program (developed at the U.S. National Institutes of Health (http://www.rsb.info.nih.gov/ij/)) and the percent density of p24 was determined by the following formula: percent density of p24 = 100 × (the density of p24 signal)/(the cumulated density of all *Gag* signals) (Tamiya et al., 2004).

3. Results

3.1. Whole capsid substitutions necessary for NFV-enhanced replication

MAmt, carrying only the substitutions in the matrix (Fig. 2), grew well in the absence of NFV (Fig. 3). In the presence of NFV, however, it did not grow at all, indicating that matrix substitutions were not sufficient to confer NFV resistance. MA + PRmt, carrying substitutions in the matrix and protease (Fig. 2), replicated as efficiently as PRmt (carrying only the substitutions in protease), both in the absence and presence of 0.1 μM NFV, though its replication was not enhanced with NFV, indicating that the substitutions in matrix and protease were not sufficient for NFV-dependent enhancement of replication. As reported in our previous study (Matsuoka-Aizawa et al., 2003), p17PRmt replicated more efficiently in the presence of 0.1 μM NFV than in the absence of NFV. Therefore, some of the substitutions in the capsid should be responsible for such unique phenotype of CL-4 strain. The HIV-1 capsid contains two domains, a C-terminal oligomerization domain and N-terminal core domain, which function differently in viral assembly (Turner and Summers, 1999). Therefore, we divided the EcoT22I—ApaI segment of CL-4 into two segments at ACC I site, named them the N-terminal half of the capsid (NCA) and the C-terminal half of the capsid (CCA), and constructed two recombinant HIV-1 clones, NCAmt and CCAmt, which possessed all the substitutions in the matrix and protease of CL-4, and the substitutions in NCA and CCA, respectively (Fig. 2). NCAmt and CCAmt grew efficiently both in the absence and presence of 0.1 μM NFV, and only NCAmt showed weak replication enhancement with 0.1 μM NFV in PM-1 and MT-2 cells though it was not so efficient as that of p17PRmt, suggesting that the substitutions in CCA, contributed to the efficient replication enhancement of p17PRmt (Fig. 3). CCAmt did not show the p17PRmt's phenotype, indicating that the substitutions in NCA were indispensable for replication enhancement. As we reported previously (Matsuoka-Aizawa et al., 2003), p24PRmt lacking the substitutions in matrix did not show replication enhancement by NFV. Taken together, the substitutions in the whole matrix, capsid, SP1, and the N-terminal end of nucleocapsid of CL-4 were indispensable for efficient replication enhancement of p17PRmt.

To define further the role of substitutions in the matrix, NCA, and CCA, viral replication efficiency was compared among the HIV-1 clones described above in the absence and presence of NFV using competitive HIV-1 replication assay (Kosalaraksa et al., 1999). MA + PRmt outgrew PRmt both in the absence and presence of 0.1 μM NFV (Fig. 4a), and MAmt was outgrown by NL4-3 in the absence of NFV (Fig. 4b), suggesting that the substitutions in the matrix of CL-4 reduced the replication of HIV-1 harboring wild-type protease, but compensated the replication of HIV-1 harboring NFV-resistant protease of CL-4. NCAmt outgrew MA + PRmt both in the absence and presence of 0.1 μM NFV (Fig. 4c), suggesting that the substitutions in NCA were compensatory for the replication of HIV-1 harboring protease and matrix of CL-4. However, CCAmt was outgrown by MA + PRmt in the absence of NFV, but its replication in the presence of 0.1 μM NFV was comparable with that of MA + PRmt under similar condition (Fig. 4d), suggesting that the substitutions in CCA reduced the replication capability of MA + PRmt, while NFV compensated the mutation effect. Sub-cloning analyses of proviral sequences at both of the passages 3 and 4 in competitive HIV-1 replication assay in the presence of 0.1 μM NFV showed that five of 10 clones were derived from CCAmt and the other five clones were derived from MA + PRmt, which confirmed that CCAmt and MA + PRmt had comparable replication ability in the presence of 0.1 μM NFV (Fig. 4d). MA + PRmt readily outgrew p17PRmt in the absence of NFV, but was outgrown by p17PR in the presence of 0.1 μM NFV (Fig. 4e), suggesting that the substitutions in NCA and CCA reduced the replication capability of MA + PRmt, while NFV counteracted the mutation effect and rather enhanced replication ability at sub-inhibitory concentration (Fig. 3, p17PRmt).

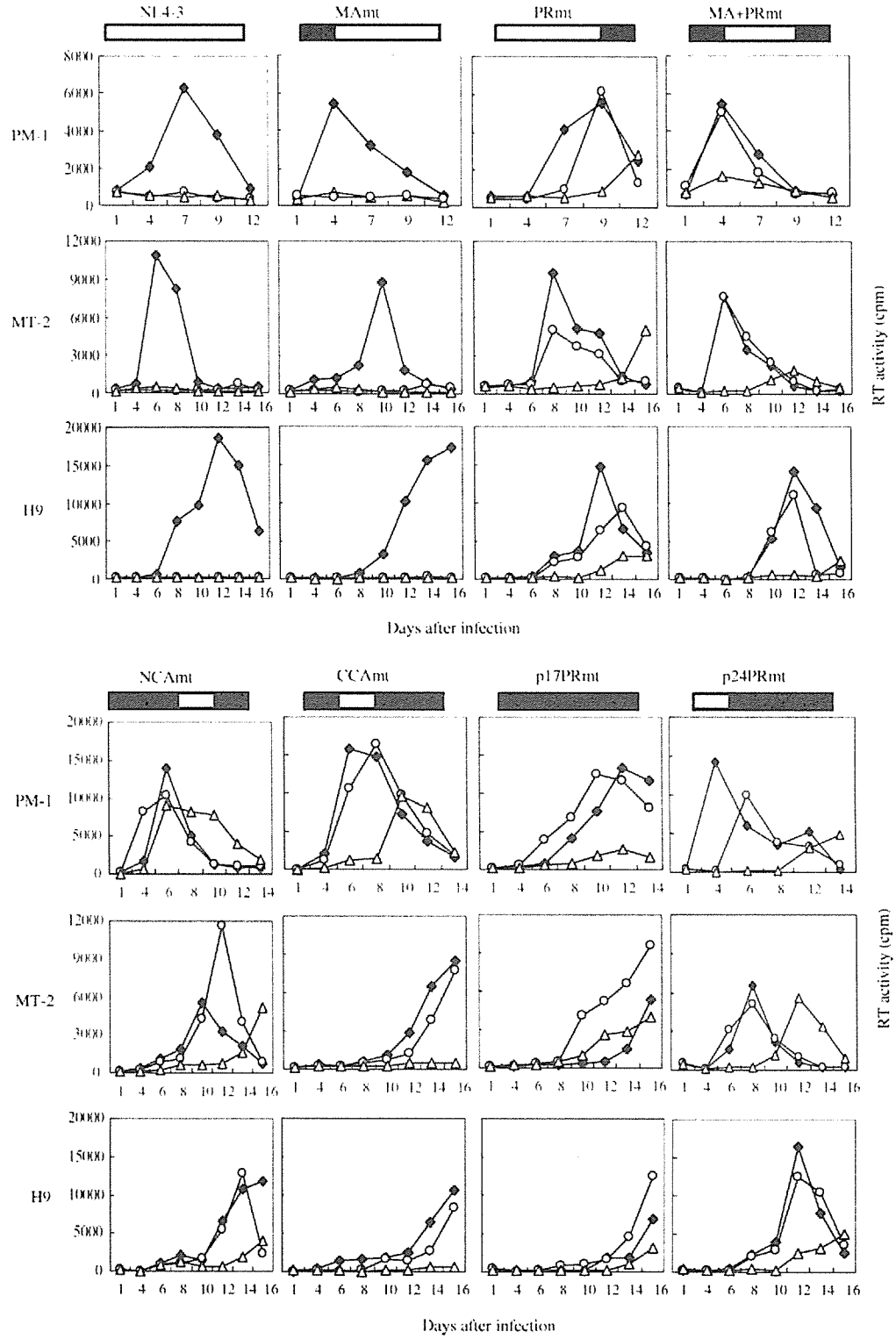

Fig. 3. Effects of NFV on replication capability of recombinant HIV-1s. PM-1, MT-2, and H9 cells (2×10^4 cells) were exposed to 0.2 ml of cell-free supernatant containing each HIV-1 clone (2×10^5 ^{32}P cpm of RT activity), washed once, and cultured in 0.2 ml of medium in the absence (closed diamonds) and presence of NFV (0.1 μM; open circles, 1 μM; open triangles). Half volume of the culture medium was changed every 2 or 3 days, and the supernatant was kept at −80 °C until the measurement of RT activity. Each experiment was carried out in duplicate and repeated three times, and representative data are shown.

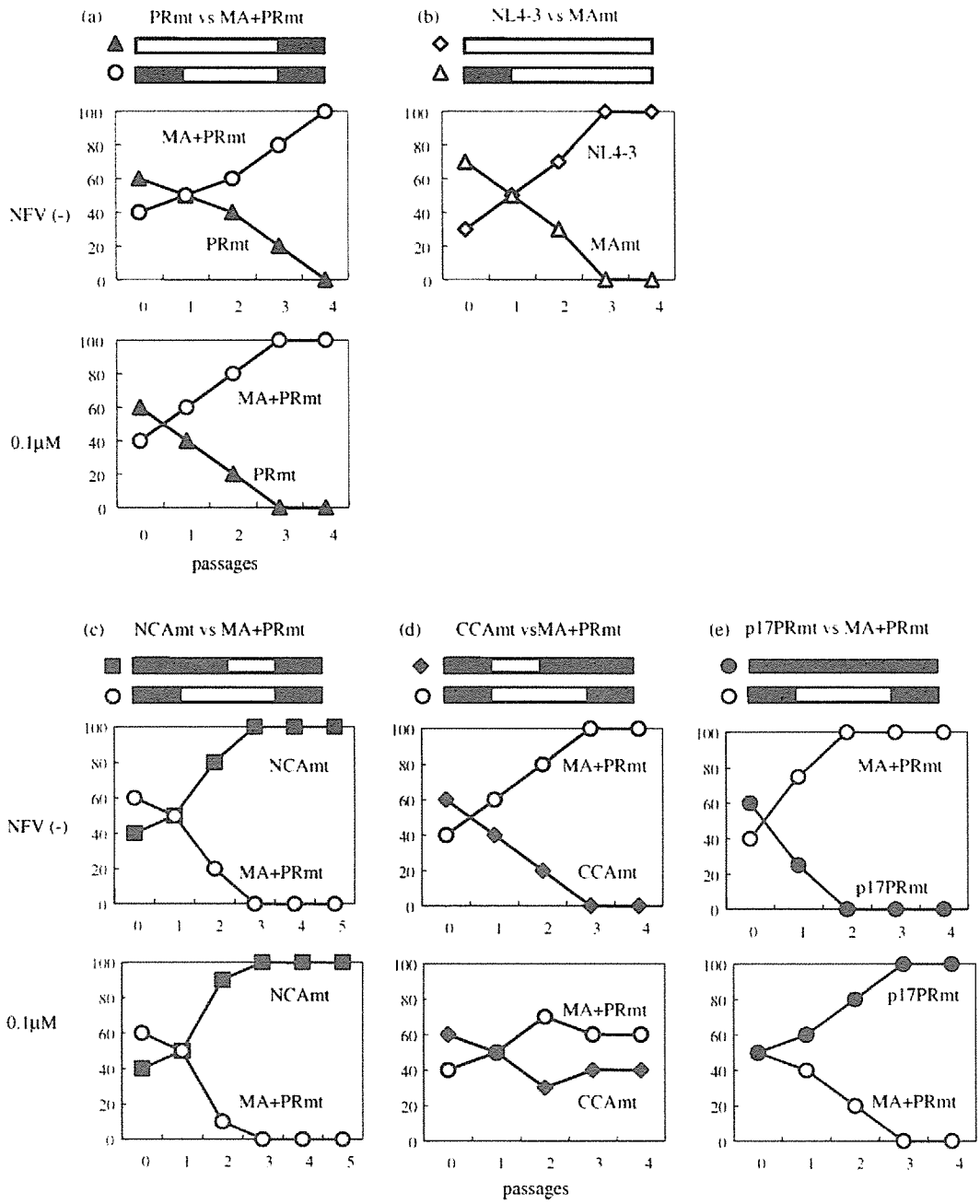

Fig. 4. One-to-one competitive HIV-1 replication assay. H9 cells (2×10^5 cells) were incubated with two recombinant HIV-1s (each of 100 $TCID_{50}$) simultaneously at 37 °C for 16 h, washed with PBS twice, and cultured in the absence and presence of 0.1 μM of NFV. At 7 days post-infection, the culture supernatant was used to infect fresh uninfected H9 cells. The cells harvested at each passage were subjected to direct DNA sequencing, and the viral population changes were determined by the relative peak height in the sequencing electrophoregram. The persistence of the original amino acid substitutions was confirmed for all infectious clones used in this assay. (a) PRmt vs. MA + PRmt; (b) NL4-3 vs. MAmt; (c) NCAmt vs. MA + PRmt; (d) CCAmt vs. MA + PRmt; (e) p17PRmt vs. MA + PRmt.

3.2. Gag substitutions conferring NFV resistance

To analyze the role of the substitutions in the matrix, NCA, and CCA in NFV resistance, IC_{50}s of NFV for the HIV-1 clones described above were determined by using MT-2 cells (Zhang et al., 1997). MAmt (IC_{50}: 9.4 nM) showed 1.9-fold resistance against NFV compared with NL4-3 (5.0 nM), and p17PRmt (893 nM) showed 3.3-fold resistance compared with p24PRmt (273 nM) (Table 1), indicating that the substitutions in the matrix may make a small contribution to the viral resistance against NFV. NCAmt (483 nM) had 2.1-fold resistance against NFV compared with MA + PRmt (229 nM), and p17PRmt had 4.1-fold resistance compared with CCAmt (217 nM), indicating that the substitutions in NCA gave positive impact on viral resistance. Interestingly, CCAmt showed 0.95-fold resistance against NFV compared with MA + PRmt, indicating that substitutions in CCA

Table 1
NFV resistance of recombinant HIV-1s

HIV-1	IC_{50} (nM)	Fold-resistance
NL4-3	5.0 ± 0.4	1.0
PRmt	241 ± 34	48
p24PRmt	273 ± 13	55
p17PRmt	893 ± 28	179
MAmt	9.4 ± 3.3	1.9
MA + PRmt	229 ± 21	46
NCAmt	483 ± 26	97
CCAmt	217 ± 32	43

The concentrations of drug added to the growth medium for calculation of the IC_{50}s were 0, 1, 3.16, 10, 31.6, 100, and 316 nM and 1 and 3.16 μM NFV, and the IC_{50}s were derived from plots of percent of inhibition of p24 production in culture supernatant versus NFV concentration.

may give small negative impact on viral resistance in the absence of the substitutions in NCA. p17PRmt, however, had 1.8-fold resistance compared with NCAmt, indicating the substitutions in CCA may give a small but positive contribution to viral resistance in the presence of the substitutions in NCA. The role of the substitutions in CCA in viral resistance was altered by the presence of the substitutions in NCA.

3.3. Gag substitutions facilitating cleavage between matrix and capsid

To further delineate the impact of each substitution, the Gag processing pattern was assessed in the absence and presence of NFV by Western blot analysis using anti-p24 monoclonal antibody (Fig. 5A1–2 and B1–2). As expected, 0.1 μM of NFV effectively blocked cleavage of the Gag p55 precursor of NL4-3 (percent density of p24; 4.7% versus 87.5% in Fig. 5A1; 4.2% versus 83.3% in Fig. 5A2). In contrast, NFV gave only a small influence on the cleavage patterns of the p55 precursor of MA + PRmt (percent density of p24; 65.5% versus 87.4% in Fig. 5A1; 77.8% versus 92.6% in Fig. 5A2), which is consistent with the indistinguishable replication kinetics of this mutant in the absence and presence of NFV (Fig. 3). Interestingly, NFV enhanced the cleavability of the p55 precursor of p17PRmt (percent density of p24; 94.8% versus 74.3% in Fig. 5A1; 72.2% versus 54.1% in Fig. 5A2), which was paralleled with NFV-dependent replication enhancement of this mutant (Fig. 3). NFV also gave a small positive effect on the cleavability of the p55 precursor of NCAmt (percent density of p24; 97.1% versus 94.6% in Fig. 5B1; 97.5% versus 96.2% in Fig. 5B2), which was paralleled with the partial enhancement of replication with NFV (Fig. 3). Furthermore, percent density of p24 of NCAmt was increased compared with that of MA + PRmt (percent density of p24; 94.6% and 96.2% versus 87.4% and 92.6% in the absence of NFV; 97.1% and 97.5% versus 65.5% and 77.8% in the presence of 0.1 μM NFV), suggesting that the substitutions in NCA play a significant role in Gag cleavability. Finally, NFV decreased percent density of p24 of CCAmt (percent density of p24; 68.9% versus 78.2% in Fig. 5B1; 45.3% versus 79.0% in Fig. 5B2), which was paralleled with NFV-induced delay of replication kinetics (Fig. 3). For further confirmation, the Gag processing pattern of NCAmt and CCAmt was also assessed by Western blot analysis using HIV-1-infected patient's serum (Fig. 5B3). As expected, NFV slightly increased cleavability of the p55 precursor of NCAmt (percent density of p24; 96.9% versus 94.5% in Fig. 5B3), and gave a negative impact on Gag cleavage of CCAmt (percent density of p24; 41.9% versus 74.3% in Fig. 5B3), which were well compatible with the cleavage pattern analyzed by using anti-p24 monoclonal antibody (Fig. 5B1, 2). In summary, NFV induced enhanced cleavability of Gag precursors of p17PRmt and NCAmt, which was well paralleled with NFV-induced enhancement of replication capability of these mutants.

4. Discussion

We previously reported that the substitutions in p6-protease segment alone are sufficient to confer NFV resistance while those in matrix are indispensable for the replication enhancement of CL-4 by NFV (Matsuoka-Aizawa et al., 2003). In the present study, we found that not only the matrix substitutions but the mutations in N-terminal half of capsid also played critical role in the enhancement and that the full potential of the enhancement phenotype was achieved only with the cooperation of mutations in the entire Gag and protease region of CL-4. The substitutions in matrix and those in N-terminal half of capsid compensated the otherwise compromised viral replication in the absence and presence of NFV (Fig. 4a and c). Probably, these substitutions cooperatively altered the tertiary structure of the Gag precursor and made the cleavage site between matrix and capsid more accessible to mutant protease harboring multiple resistance-associated mutations. The cleavage pattern analyzed by Western blot analysis supported the idea that the substitutions in N-terminal half of capsid improved the Gag cleavage. Percent density of p24 of NCAmt was increased compared with that of MA + PRmt in the absence of NFV (Fig. 5A1–2 and B1–2; 94.6% and 96.2% versus 87.4% and 92.6%). It is worth noting that CL-4 had a total of 56 amino acid substitutions in *gag-pro* genes compared with NL4-3; 22 substitutions had emerged during NFV-containing therapy, and 34 other substitutions had already existed before the introduction of the therapy, and that all the substitutions in N-terminal half of capsid of CL-4 were pre-existing before NFV-therapy (Fig. 1), suggesting that certain polymorphic amino acid residues seen in HIV-1 clinical isolates were associated with drug resistance. Interestingly, the amino acid insertion at the same site of the matrix of CL-4 compared with NL4-3 (Fig. 1; amino acids 121–125 in MA, QQAAA) was reported to increase viral replication harboring mutant protease by improving otherwise impaired Gag processing (Tamiya et al., 2004). Gatanaga et al. also reported that a polymorphic substitution in N-terminal half of capsid was indispensable for the development of high multitude of resistance against PIs (Gatanaga et al., 2002), though CL-4 did not harbor the same substitution. It is also known that certain drug-resistance-conferring amino acid substitutions found in one subtype HIV-1 isolated from patients under therapy may be detected in HIV-1 of other subtypes from untreated individuals (Cornelissen et al., 1997; Quinones-Mateu et al., 1998). More-

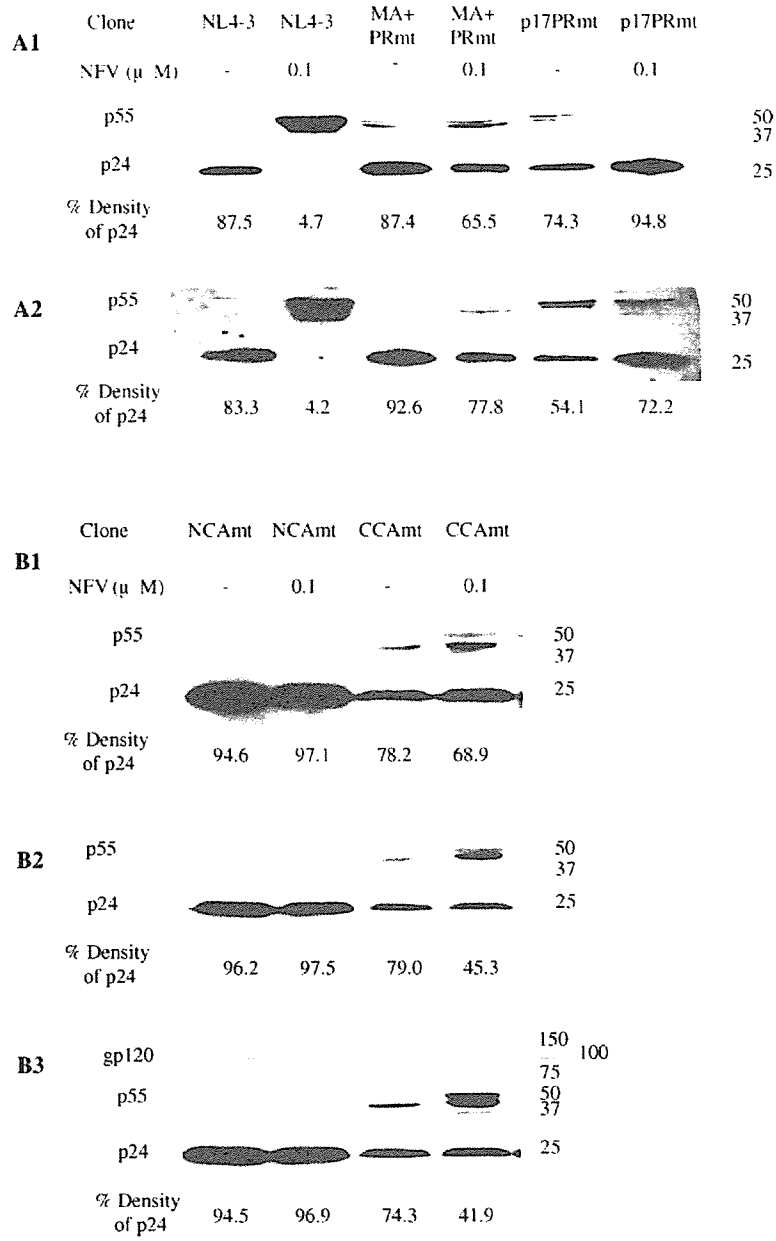

Fig. 5. Western blot analysis in the absence and presence of NFV. HeLa cells were transfected with each of full-length molecular clones and cultured in the absence and presence of 0.1 μM NFV. At 48 h post-transfection, virions in the culture supernatant (6×10^5 cpm of RT activity) were harvested and subjected to Western blot analysis. Gag proteins were visualized by using anti-p24 monoclonal antibody (A1-2, B1-2) and HIV-1-infected patient's serum (B3). Percent density of p24 was calculated as 100 × (p24 signal density/total Gag product signal densities) in a Western blot.

over, a recent study of Colson et al. revealed that HIV-2 strains harbor specific patterns of natural polymorphism and resistance (Colson et al., 2004). HIVs seem to acquire drug-resistance by utilizing the pre-existing polymorphic mutations and by coordinating the development of multiple substitutions.

Furthermore, the substitutions in N-terminal half of capsid of CL-4 altered the effect of NFV on viral replication. Sub-inhibitory concentration (0.1 μM) of NFV slightly accelerated the Gag precursor cleavage of NCAmt (percent density of p24; 97.1% versus 94.6% in Fig. 5B1; 97.5% versus 96.2% in Fig. 5B2; 96.9% versus 94.5% in Fig. 5B3), which was paralleled with the partial replication enhancement with NFV (Fig. 3), though it showed inhibitory effect in Gag processing of MA + PRmt (percent density of p24; 65.5% versus 87.4% in Fig. 5A1; 77.8% versus 92.6% in Fig. 5A2). Therefore, one of the mechanisms of viral replication enhancement with NFV is the improved processing of Gag harboring the substitutions in N-terminal half of capsid of CL-4 cooperated with the substitutions in the matrix. On the other hand, the role of the substitutions in C-terminal half of capsid seemed different, though they were also indispensable for the full potential of replication enhancement with NFV. They impaired the cleavability of Gag precursor

of MA + PRmt (Fig. 5A1–2 and B1–2; percent density of p24; CCAmt versus MA + PRmt = 78.2% and 79.0% versus 87.4% and 92.6%) and NCAmt (Fig. 5A1–2 and B1–2; percent density of p24; p17PRmt versus NCAmt = 74.3% and 54.1% versus 94.6% and 96.2%) in the absence of NFV, which were parallel with viral replication data (CCAmt versus MA + PRmt, Fig. 4d; p17PRmt versus NCAmt, Fig. 3). The effects of NFV on Gag cleavage pattern were different between CCAmt and p17PRmt; sub-inhibitory concentration (0.1 μM) of NFV facilitated the Gag cleavability of p17PRmt (percent density of p24; 94.8% versus 74.3% in Fig. 5A1; 72.2% versus 54.1% in Fig. 5A2), though it decreased the cleavability of CCAmt Gag (percent density of p24; 68.9% versus 78.2% in Fig. 5B1; 45.3% versus 79.0% in Fig. 5B2; 41.9% versus 74.3% in Fig. 5B3), which was also parallel with viral replication data showing enhancement only in p17PRmt but not in CCAmt (Fig. 3). Considering together, the substitutions in C-terminal half of capsid compromised viral replication by impairing the Gag preprocessing, and NFV could counteract the negative impact only in the presence of the substitutions in N-terminal half of capsid. In the absence of the substitutions in N-terminal half of capsid, only partial counteraction was seen (Fig. 4d). In summary, NFV-induced viral replication enhancement of CL-4 was caused by two mechanisms; NFV facilitates the processing of Gag harboring the substitutions in the matrix and N-terminal half of capsid of CL-4, and NFV counteracts the impaired Gag cleavage caused by the substitutions in C-terminal half of capsid of CL-4 only in the presence of the substitutions in the matrix and N-terminal half of capsid of CL-4. Therefore, the full potential of the enhancement phenotype was achieved only with the cooperation of mutations in the entire Gag and protease region of CL-4.

Notably, we found several other PI-resistant isolates with the phenotype of PI-dependent replication enhancement (data not shown), suggesting that HIV-1 can evolve to acquire capability to replicate better with the drugs. Such replication enhancement with antiretroviral agents presents formidable challenge in the therapy of HIV-1 infection. Future studies of structural analyses of Gag precursor(s) harboring substitutions of these mutants are warranted to clarify the underlying mechanism(s).

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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

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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.
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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-

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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)	

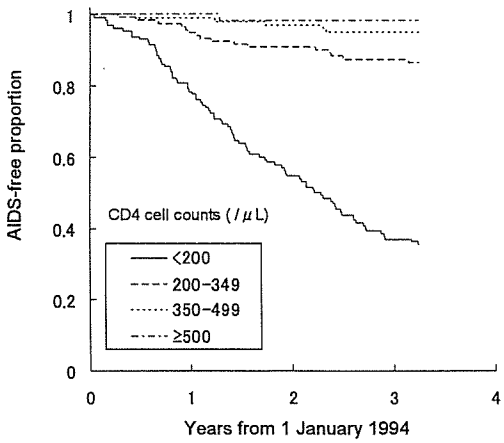

Figure 1. AIDS-free proportion between January 1, 1994 and March 31, 1997 by CD4 cell count in the first quarter of 1994.

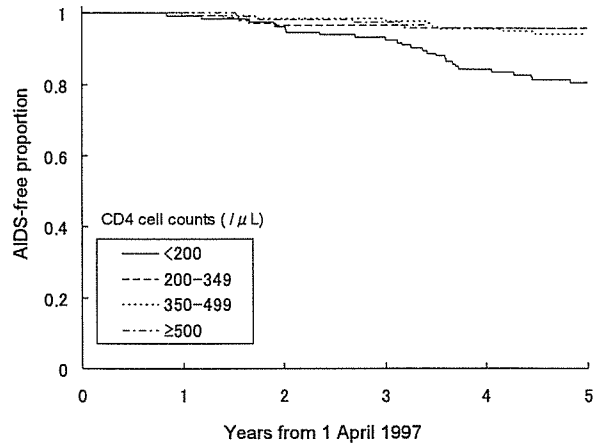

Figure 2. AIDS-free proportion between April 1, 1997 and March 31, 2002 by CD4 cell count in the second quarter of 1997.

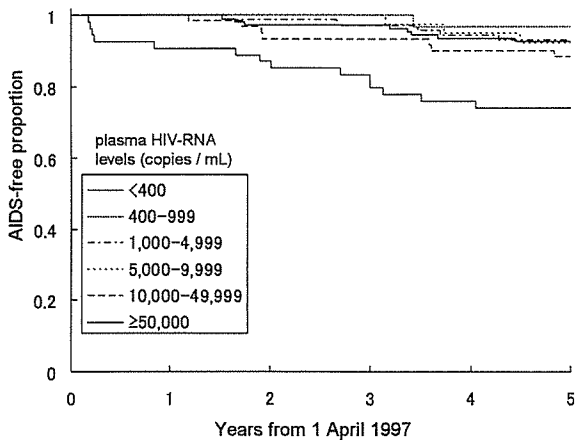

Figure 3. AIDS-free proportion between April 1, 1997 and March 31, 2002 by plasma HIV RNA level in the second quarter of 1997.

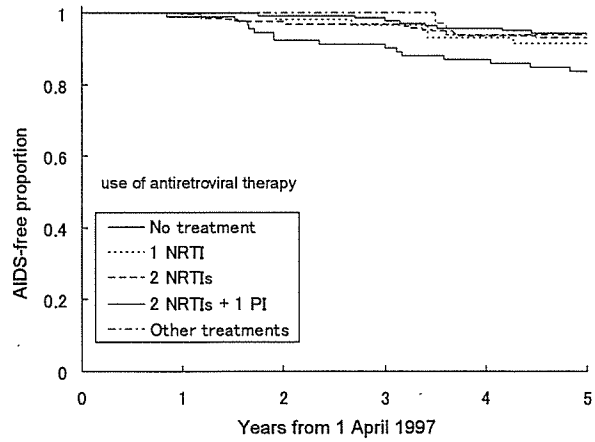

Figure 4. AIDS-free proportion between April 1, 1997 and March 31, 2002 by the use of antiretroviral therapy in the second quarter of 1997.

* 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.[5]

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.

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Original article

Decrease in Epstein–Barr virus-positive AIDS-related lymphoma in the era of highly active antiretroviral therapy

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Abstract

Recent introduction of highly active antiretroviral therapy (HAART) is reported to have reduced the incidence of lymphoma among HIV-infected individuals. A clinicopathological study was performed on 86 AIDS-related lymphoma patients who were treated in Tokyo area from 1987 to 2005. The incidence of lymphoma detected by autopsy was 27% (53 cases/198 autopsies). Diffuse large B cell lymphoma was the most predominant histological subtype throughout the period (78%). Burkitt's lymphoma (BL) increased from 2% in the pre-HAART era (before end-1997) to 13% in the HAART era, whereas incidence of BL did not vary between HAART users and non-users. Epstein–Barr virus (EBV)-positive lymphoma decreased from 88% in the pre-HAART era to 58% in the HAART era, but did not differ significantly between HAART users (73%) and non-users (74%). Nodal involvement of lymphoma increased from 14% in the pre-HAART era to 50% in the HAART era; however, central nervous system involvement decreased from 62 to 38%. Kaposi's sarcoma-associated herpesvirus infection was rare (4%) among all cases. These data suggest that HAART might play a partial role in these changes, and the alteration in immunological backgrounds, such as EBV prevalence, is suggested as another leading cause of these changes in Japanese AIDS-related lymphoma.

Keywords: AIDS-related lymphoma; Burkitt's lymphoma; Diffuse large B cell lymphoma; Epstein–Barr virus; Highly active antiretroviral therapy

1. Introduction

Patients with human immunodeficiency virus type 1 (HIV-1) infection suffer from an elevated risk of malignancies during the course of their disease [1–3]. Lymphoma, as well as Kaposi's sarcoma, is among the common AIDS-related malignancies [4,5]. The risk of lymphoma has been reported to be at least 60 times higher in HIV-1-infected patients than in the general population [6]. Epstein–Barr virus (EBV) is known to play an important role in the pathogenesis of AIDS-related lymphoma [4]. EBV has been detected in more than 70% of cases of AIDS-related lymphoma in Japan [7,8].

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EBV-positive AIDS-related lymphoma cells frequently express EBV-encoded oncoproteins such as latent membrane protein 1 (LMP-1) and EBV-encoded nuclear antigens [4,7,8]. Thus, a large number of AIDS-related lymphoma cases were categorized into EBV-associated opportunistic lymphoma in Japan. Kaposi's sarcoma-associated herpesvirus (KSHV; human herpesvirus 8, HHV-8) is another causative agent for the virus-associated opportunistic lymphoma in patients with AIDS [4]. Primary effusion lymphoma (PEL) and some cases of solid lymphoma are associated with KSHV infection [9–11].

The recent advent of highly antiretroviral therapy (HAART) has drastically altered the natural history of HIV-1 infection, by reducing various opportunistic diseases and improving the underlying immune deficiency [12–15]. Indeed, it has dramatically decreased mortality in HIV-1-infected patients [16]. However, recent articles suggest that the magnitude of reduction in lymphoma incidence appears to be less significant [1,12,15,17–23]. Most recently published studies originate from industrialized countries, including the U.S. and Europe, while there have been only a few reports about the clinicopathological features of large series of AIDS-related lymphoma from Asian countries [24,25]. East Asia is a high endemic area of EBV and many cases of EBV-associated diseases, such as nasopharyngeal carcinoma, gastric cancer and lymphoma, have been reported in this area [26–29]. The aim of the current study was to clarify the clinicopathological characteristics of AIDS-related lymphoma in Japan, with an emphasis on the comparison of such characters between the pre-HAART and HAART era.

2. Patients and methods

2.1. Patients

Eighty-six cases of AIDS-related lymphoma were investigated retrospectively (Table 1). These patients were referred to three hospitals in Tokyo: Tokyo Metropolitan Komagome Hospital; Research Hospital, the Institute of Medical Science, the University of Tokyo; and International Medical Center of Japan. Since a large number of AIDS patients in Tokyo area have visited the three hospitals, more than half of all AIDS-related lymphoma cases in Tokyo area were covered in the present study. We reviewed all cases diagnosed histologically as AIDS-related lymphoma from January 1987 to March 2005. Fifty-three specimens obtained from autopsy and 33 biopsy specimens were studied. The histological sub-typing of lymphoma was based on the World Health Organization (WHO) classification [30]. Particularly, the diagnosis of Burkitt's lymphoma (BL) was based on histology and chromosome data, as recommended in the WHO classification [30]. Clinical data, such as age, sex, risk factor, CD4 cell count, use of HAART and prognosis, were collected from the medical records. As for the CD4 cell count, the counts at the time of lymphoma diagnosis were used.

In this study, HAART was defined as prescription of at least three antiretroviral drugs, including a protease inhibitor or a non-nucleoside reverse transcriptase inhibitor. Central nervous system (CNS) and lymph node (LN) involvement of lymphoma were determined according to the autopsy records. HAART was introduced in Japan in 1996–1997. Thus, to assess the effects of HAART, we introduced two criteria as follows: era and individual therapeutic method. First, we divided 86 patients on the basis of their date of diagnosis into two calendar periods: 1987–1997, defined as the pre-HAART era and 1998–2005, defined as the HAART era. Second, to characterize more specifically, we divided cases into HAART users who received HAART before diagnosis of lymphoma and HAART non-users who did not.

2.2. Methods

Cell lineage on each case was determined by immunohistochemistry as described previously [11]. Anti-CD3 (DAKO,

Table 1
Characteristics of patients diagnosed with AIDS-related lymphoma

	All patients ($n=86$)	Pre-HAART era (1987–1997) ($n=47$)	HAART era (1997–2005) ($n=39$)	P	HAART(−) ($n=71$)	HAART(+) ($n=15$)	P
Histology (DLBCL/BL/HD/PEL/Poly/ATLL/unknown)	67/6/3/1/2/1/6	37/1/1/1/1/0/6	30/5/2/0/1/1/0	–	56/5/1/1/2/0/6	11/1/2/0/0/1/0	–
Histology (% DLBCL/BL/other/unknown)	78/7/8/7	79/2/6/13	77/13/10/0	–	79/7/5/8	73/7/20/0	–
Sex (% female)	4.6%	6.4%	2.6%	0.382 (F)	4.2%	6.7%	0.861 (F)
Age (year), median (mean, range)	42 (42.4, 12–74)	41 (41.3, 12–74)	43 (43.7, 25–64)	0.294 (MW)	41 (41.4, 12–74)	50 (47.2, 29–63)	0.082 (MW)
CD4 (cells/μl), median (mean, range)	25.5 (89.8, 0–519)	8 (57.2, 0–496)	75 (129.1, 0–519)	<0.01 (MW)	20 (70.5, 0–496)	154 (181, 0–519)	<0.01 (MW)
EBER (%, positive)	73.7%	87.5%	58.3%	<0.01 (C)	73.7%	73.3%	0.650 (F)
KSHV, cases (%)	4 (4.6%)	3 (6.4%)	1 (2.6%)	0.371 (F)	4 (5.6%)	0 (0%)	0.458 (F)
Risk factor (% sexual)	87.2%	82.9%	92.3%	0.167 (F)	87.3%	86.7%	0.612 (F)
Prognosis (% death)	73.2%	87.2%	56.4%	<0.01 (C)	62.0%	60.0%	0.887 (C)
CNS involvement[a] (%)	54.7%	62.2%	37.5%	0.098 (C)	57.7%	37.5%	0.249 (F)
LN involvement[a] (%)	25.5%	14.2%	50.0%	0.010(F)	23.3%	37.5%	0.384 (F)

P values were calculated by Chi-square test (C), Fisher's exact (F), and Mann–Whitney test (MW). ATLL, adult T cell leukemia/lymphoma; HD, Hodgkin lymphoma; Poly, polymorphic B cell lymphoma.

[a] CNS and LN involvements were investigated only by autopsy.

Copenhagen, Denmark) and/or anti-CD45RO (DAKO) antibodies for T cell and anti-CD20 antibody (DAKO) for B cell were used as primary antibodies. Presence of EBV was examined by in situ hybridization for EBV-encoded small RNAs (EBER), as described previously [31]. KSHV was detected by immunohistochemistry using antibody against KSHV-encoded latency-associated nuclear antigen (LANA) [32]. Analysis of statistical significance was carried out using the Chi-square test or Fisher's exact for bivariate tabular analysis and Mann–Whitney test for comparison of two independent groups of sampled data, such as CD4 cell counts.

3. Results

3.1. Characteristics of AIDS-related lymphoma

The incidence of lymphoma detected by autopsy in AIDS patients was 27% (53 cases/198 autopsies) from January 1987 to March 2005. Together with 33 biopsies, the characteristics of the whole 86 cases of AIDS-related lymphoma are summarized in Table 1. The study group included 82 men and four women with a mean age of 42.4 years (range, 12–74 years). HIV-1 transmission occurred via sexual contact in 87% of cases and injecting drug use in only one case. The majority of the other patients were hemophiliacs (8%). According to the WHO classification criteria, the cases of AIDS-related lymphoma were categorized into several different types of lymphoma [13,30]. In general, Japanese cases of AIDS-related lymphoma have been classified into two major types as follows: diffuse large B cell lymphoma (DLBCL) and BL. Of the 86 cases, 67 (78%) were DLBCL and six (7%) were BL (Fig. 1a). Clinicopathological data of the two types are detailed in Tables 2 and 3. The other rare types were Hodgkin lymphoma, polymorphic B cell lymphoma, PEL and adult T cell lymphoma/leukemia. Three cases of Hodgkin lymphoma consisted of two cases of mixed cellular subtype and one case of lymphocyte depletion subtype. CNS involvements was found in 55% of autopsy cases, of which 26% involved the CNS solely. Twenty-five percent of patients had LN involvement. EBER was detected in 74% of cases (Fig. 1b). The KSHV-encoded LANA was identified in four cases of solid lymphoma (5%). One of those cases was complicated with KSHV-positive PEL. Two of those cases also contained EBV in addition to KSHV. The characteristics

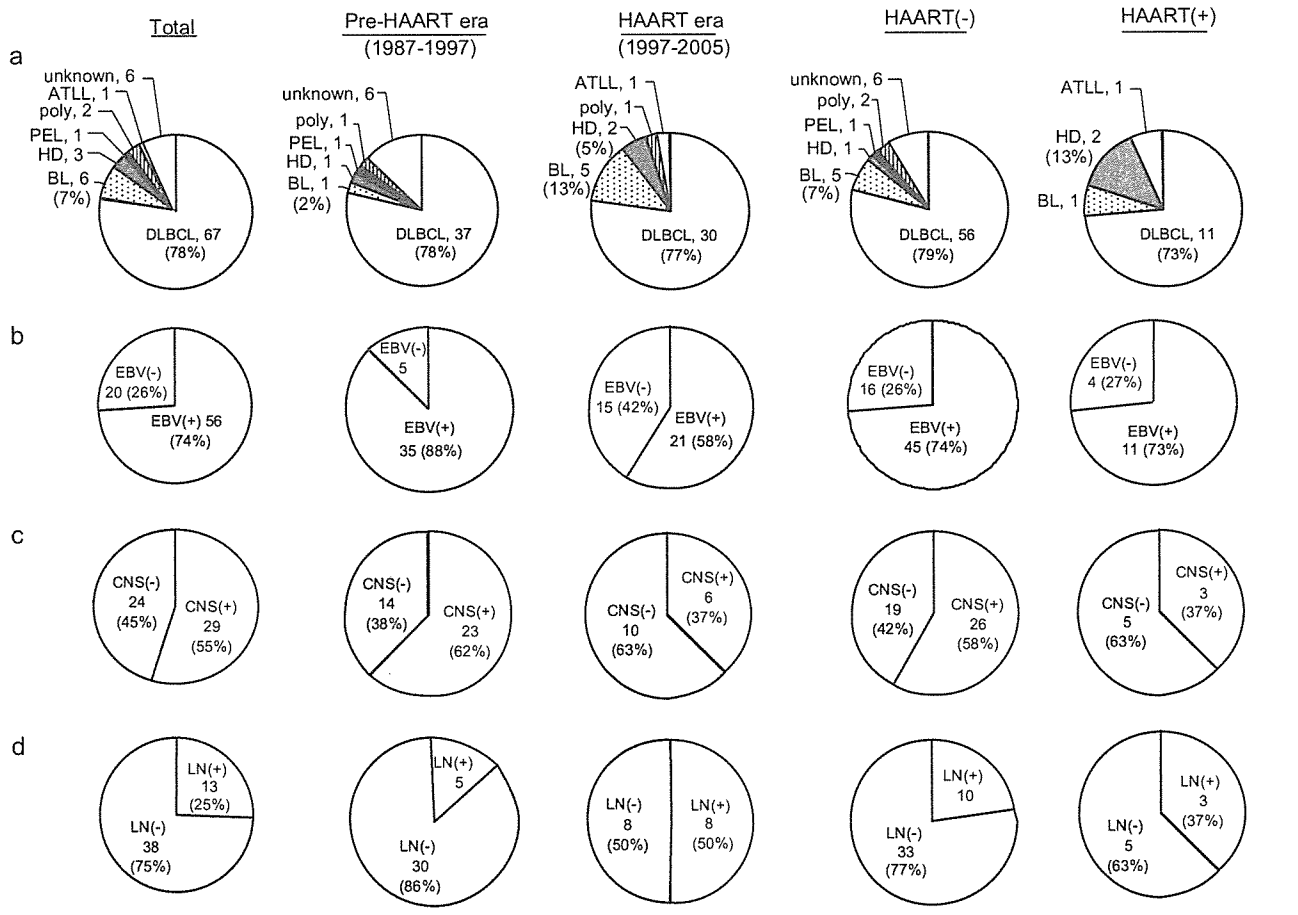

Fig. 1. Comparison of histological subtypes, EBV-positive rate, CNS and LN involvements. Histological subtypes (a), EBV-positive rate (b), CNS (c) and LN involvements (d) were compared for AIDS-related lymphoma in Tokyo area. Each number indicates the number of cases. Percentages are indicated in parentheses. See Table 1 for abbreviations.