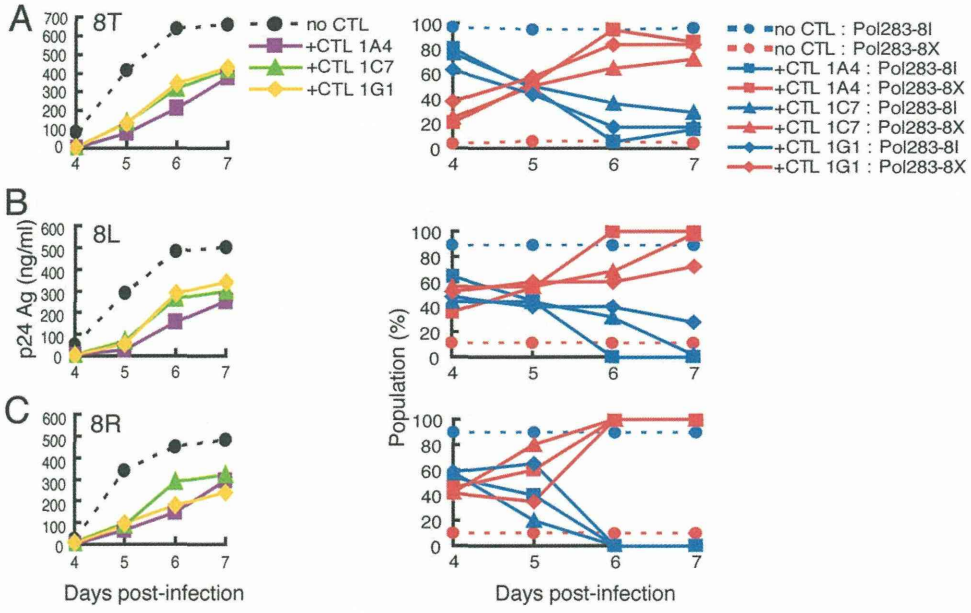

FIG. 4. *In vitro* selection of Pol283 escape mutants by a Pol283-8-specific CTL clone. T1 cells were infected with paired viruses (NL-432 [Pol283-8I] and a mutant virus [Pol283-8L, -8T, or -8R]) at a ratio of 9:1. The infected cells were incubated with Pol283-8-specific CTL clones at an E:T ratio of 1:0.05. The population change in the viral mixture was determined by the relative peak height on the sequencing electrogram. From day 4 to day 7 postinfection, culture supernatants were collected, and the concentration of p24 Ag in these supernatants was measured by an ELISA. The data obtained by using the mixture of Pol283-8T, -8L, or -8R with Pol283-8I are shown in panels A, B, and C, respectively.

Pol283-8T-specific CD8$^+$ T cells after the Pol283-8T mutation appeared. None of 4 HLA-B*5101$^+$ hemophiliac donors carrying Pol283-8T (KI-032, KI-121, and KI-127 [Table 2] and 1 ART-treated hemophilic donor, KI-078 [data not shown]) had detectable Pol283-8-specific CD8$^+$ T cells by analysis using the specific tetramers. But they may have had very small numbers of memory CD8$^+$ T cells. To induce Pol283-8-specific CD8$^+$ T cells from a possible Pol283-8-specific memory T-cell source, we stimulated PBMCs from these patients with the Pol283-8 peptide and then measured the number of Pol283-8-specific CD8$^+$ T cells in 2-week cultures. The KI-127 and KI-078 cultures indeed showed the presence of Pol283-8-specific CD8$^+$ T cells, but KI-127 lost the detectable memory response by April 2006 (Fig. 5), indicating that these 2 patients could maintain Pol283-8-specific memory CD8$^+$ T cells for more than 20 years. In contrast, Pol283-8T-specific CD8$^+$ T cells were not detected among PBMCs from any of these 4 donors after 2 weeks in culture (Fig. 5), indicating that the Pol283-8T escape mutant did not elicit specific CD8$^+$ T cells *in vivo*. These results support the idea that the Pol283-8T mutant was selected by Pol283-8-specific CTLs in donors first infected with the wild-type virus. Similarly, Pol283-8R-specific CD8$^+$ T cells were not detected in KI-007, although this patient had Pol283-8-specific memory CD8$^+$ T cells (Fig. 5), supporting the notion that the 8R mutant was an escape mutant selected by Pol283-8-specific CTLs and failed to elicit these escape mutant-specific CTLs.

DISCUSSION

It is well known that HLA-B*57 and -B*27 are associated with slow progression to AIDS (19, 37). HLA-B*57-mediated and HLA-B*27-mediated effects on disease progression are seen early and late, respectively, during an infection (6, 14). In the present study, we analyzed 108 HIV-1-infected Japanese hemophiliacs. In Japan, 1,439 patients had been infected with HIV-1 before 1985, mostly around 1983. At present, only 801 of these patients remain alive. Since they had not been treated with highly active antiretroviral therapy (HAART) before 1997, the survivors would seem to be slow progressors. This cohort does not include a large number of patients, because it is not easy to recruit a large number of HIV-1-infected hemophiliacs in Japan, where only 800 are still alive. We found that HLA-B*5101 had effects on the slow progression of the disease in the late phase (both in 1998 and during the years from 1998 to 2007), even when a small number of samples was analyzed. Our recent study also revealed that HLA-B*5101$^+$ hemophiliacs had lower VLs and higher CD4 counts than HLA-B*5101$^-$ hemophiliacs but that only the CD4 count was significantly higher in HLA-B*5101$^+$ than in HLA-B*5101$^-$ hemophiliacs (20). These findings support the idea that HLA-B*5101-restricted immune responses are associated with slow progression to AIDS.

Pol283-8, Pol743-9, and Gag327-9 are thought to be immunodominant HIV-1 epitopes, because CTLs specific for them were frequently detected in chronically HIV-1 infected HLA-B*5101$^+$ individuals (45). A previous study demonstrated that Pol283-8-specific and Pol743-9-specific CTLs suppress HIV-1 replication strongly but that Gag327-9-specific CTLs suppress it only weakly *in vitro* (43), suggesting that HIV-1 replication can be suppressed *in vivo* by Pol283-8-specific and Pol743-9-specific CTLs. In the present study, we demonstrated that a higher number of Pol283-8-specific CD8$^+$ T cells was detected predominantly in LTNPs, whereas Pol743-9-specific CD8$^+$ T cells were found at higher levels in all 10 of the SP hemophiliac

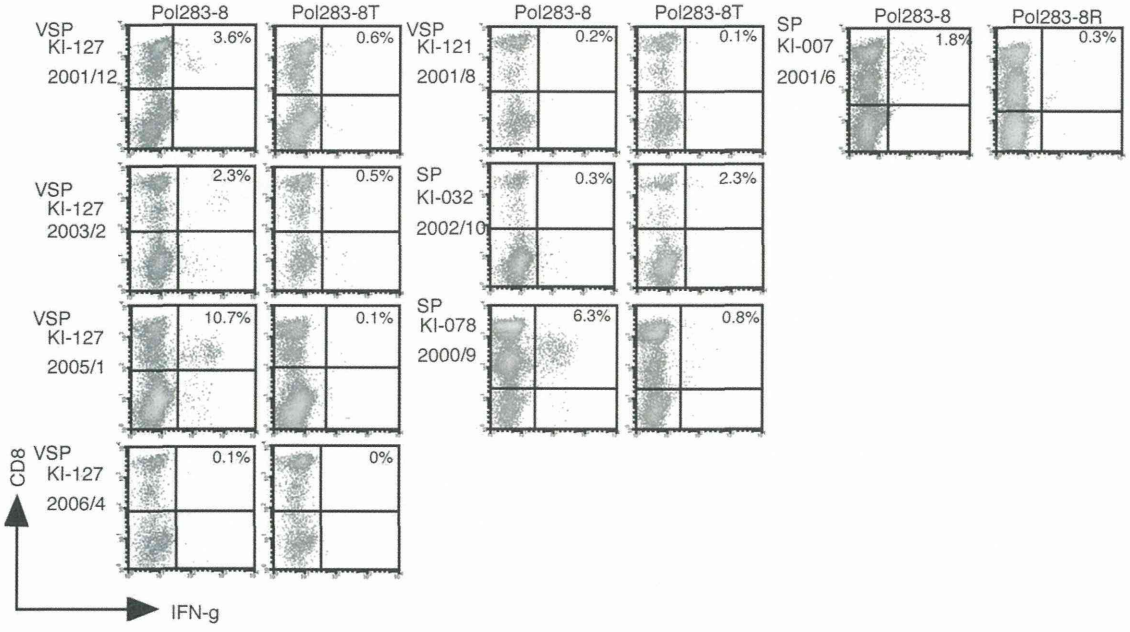

FIG. 5. Induction of Pol283-8-specific CD8+ T cells from PBMCs of 2 very slow progressors and 3 slow progressors. PBMCs from 2 very slow progressors (KI-127 and KI-121) and from 3 slow progressors (KI-032, KI-007, and KI-078) were stimulated with the Pol283-8 epitope peptide or the Pol283-8T or -8R peptide and were then cultured for 12 to 14 days. The cultured cells were stimulated with C1R-B*5101 cells prepulsed with the peptide. IFN-γ-producing CD8+ T cells were measured by using flow cytometry. The percentages of IFN-γ-producing CD8+ T cells are given in the upper right quadrants.

patients examined. ART-treated HLA-B*5101+ patients also carried Pol743-9-specific CD8+ T cells but not Pol283-8-specific CD8+ T cells (data not shown). The frequency of Pol283-specific CD8+ T cells was negatively correlated with the pVL, whereas the frequencies of the other 3 types of T cells were positively correlated with the pVL (Fig. 3). The longitudinal analysis of KI-127 showed that the VL increased after the 8T mutant appeared. This suggests that Pol283-specific CTLs may control HIV-1 in this patient, but the possibility that other CTLs also control HIV-1 cannot be excluded. These results support the notion that Pol283-8-specific CTLs play a key role in the control of HIV-1 in chronically HIV-1 infected HLA-B*5101+ hemophiliacs.

Previous studies showed that Gag-specific responses are negatively correlated with VL in chronically HIV-1 infected individuals (23, 25, 28, 49). Especially HLA-B*57/5801-, HLA-B*27-, HLA-B*13-, or HLA-B*63-restricted Gag-specific CD8+ T-cell responses are related to a low viral load (12, 16, 23, 34, 49). However, these studies had been performed with Caucasian and African cohorts. Since HLA-B*57/5801, HLA-B*27, and HLA-B*13 are very rare in Japan, Gag-specific CD8+ T-cell responses might not be related to a low pVL in Japanese patients. For the HLA-B*5101+ hemophiliacs studied here, it is striking that Pol283-specific CD8+ T-cell responses were much more effective in the control of HIV replication than Gag327-specific CD8+ T-cell responses. A previous study revealed that simian immunodeficiency virus (SIV)-infected cells are recognized earlier by Pol-specific T cells than by Nef-specific T cells (39). These results suggest that Pol-specific responses may be important in the control of HIV-1, and not only in the Japanese population. This is potentially an important result in relation to vaccine design and the specificity of the CD8+ T-cell responses that must be induced to achieve immune control of HIV.

Our recent study using 9 cohorts showed that there are 4 mutations (8T, 8R, 8L, and 8V) at position 8 of the Pol283 epitope, that the frequency of the 8T variant is significantly higher in HLA-B*5101+ donors than in HLA-B*5101− donors, and that some acutely infected HLA-B*5101+ subjects who had been infected with the wild-type virus had the 8T virus at only 6 or 12 months after the first test (20), indicating that the 8T mutant is selected by Pol283-specific CTLs. In the present study, we revealed that the Pol283-8T escape mutation was detected for the first time approximately 20 years post-HIV-1 infection in KI-127, indicating that this mutation had been slowly selected by Pol283-8-specific CTLs in this donor. Pol283-8R and Pol283-8L were also apparently escape mutants, because Pol283-8-specific CTLs failed to suppress the replication of HIV-1 carrying these mutants. However, the frequency of these mutations is not significantly higher in HLA-B*5101+ donors than in HLA-B*5101− donors (20), suggesting that other, non-HLA-B*5101-restricted CTLs may also select these particular mutants. Nonetheless, it is clear that the HLA-B*5101-restricted Pol283-specific CTLs select the 8R mutant, because KI-007, who had the 8R mutant virus, possessed Pol283-specific memory T cells (Fig. 5), and one HLA-B*5101+ subject with an acute HIV infection who had been infected with the wild-type virus had the 8R mutant 12 months after the first test (20).

The Pol283-8V mutant was found in only 6 of 60 HLA-B*5101+ donors, including 3 LTNP hemophiliacs (data not shown). Of the 3 nonhemophiliacs, 2 were progressors and 1 was a slow progressor. Since this mutation is rare and it is speculated that the mutations had not accumulated 25 years

ago, it is unlikely that the 3 LTNP hemophiliacs had been infected with this mutant virus. On the other hand, the 3 nonhemophiliacs may have been infected with the 8V mutant. The 8V mutation did not influence the killing activity of Pol283-8-specific CTLs toward target cells infected with the HIV-1 mutant, whereas the ability of CTLs to suppress replication was significantly weaker for the Pol283-8V mutant than for the wild-type virus. Previous studies showed that HIV-1-specific CTL clones can partially suppress HIV-1 replication but fail to kill HIV-1-infected $CD4^+$ T cells (42, 45), indicating that the replication suppression assay is more sensitive than the CTL assay. Since Pol283-8-specific CTLs cannot completely suppress the replication of the 8V mutant virus, and since the 8V virus has a higher fitness cost than the wild-type virus, the donors selecting this mutant virus can be LTNP hemophiliacs. However, it still remains unclear why the 8V virus appears in both LTNPs and progressors. We are now analyzing the HLA-B^*5101^+ nonhemophiliacs carrying the 8V mutants in order to compare them with the LTNPs carrying the 8V mutant.

Our previous study on the crystal structure of the HLA-B*5101–Pol283-8 peptide complex showed that the C-terminal anchor (PC) pocket is hydrophobic and relatively small compared with those of the serologically close alleles, HLA-B*3501 and -B*5301, whose C-terminal preferential amino acids include aromatic amino acids (30). Those findings explain why the PC residues for HLA-B*5101 are preferably aliphatic amino acids and not bulky aromatic amino acids. The PC residue is tethered with well-ordered polar and hydrophobic interactions, as observed in other major histocompatibility complex (MHC) class I molecules (Fig. 6A). Thus, the amino acid substitutions of the PC residue did not likely lead to large rearrangements of this network, and so the orientations of the side chains were presumably maintained. In the case of the 8R mutation, the PC pocket was not large enough to accommodate the Arg residue (Fig. 6B), conferring structural changes around the PC pocket that could possibly result in a lack of binding activity toward HLA-B*5101 (2). The 8L mutant exhibited slightly reduced binding activity toward HLA-B*5101 and CTL recognition for 8L peptide-pulsed target cells but no CTL response to 8L mutant-infected cells, suggesting that the mutation had a deleterious effect on antigen presentation in the system for export to the cell surface. The 8V mutation would delete only one methylene group from the Ile residue and thus would presumably have only a small influence on the binding to HLA-B*5101 as well as on its specific T-cell receptor (TCR) recognition. On the other hand, the Pol283-8T mutation likely introduces a hydrophilic OH group that probably is not appropriate for the hydrophobic pocket, resulting in diminished binding activity (43). Furthermore, the Pol283-8T mutation was detrimental to the CTL response and thus may also have induced a structural rearrangement that had a negative effect on TCR recognition.

A higher accumulation of Pol283-8 escape mutations is found in the Japanese population than in other populations, because the frequency of HLA-B*51 is much higher in Japan than in other countries (20). The fitness of the 8T, 8R, and 8L viruses is similar to that of the wild-type virus, and these escape mutants do not revert to wild-type viruses in $HLA\text{-}B^*5101^-$ donors (20). The donors with escape mutant viruses failed to elicit escape mutant-specific CTLs. These findings suggest a difficulty in controlling the replication of these mutant viruses in $HLA\text{-}B^*5101^+$ individuals initially infected with the mutant virus. We showed previously that recently infected $HLA\text{-}B^*5101^+$ donors have no advantage in the control of HIV-1 (20). Thus, the association between HLA-B*5101 and slow progression to AIDS may disappear in newly HIV-1 infected Japanese donors.

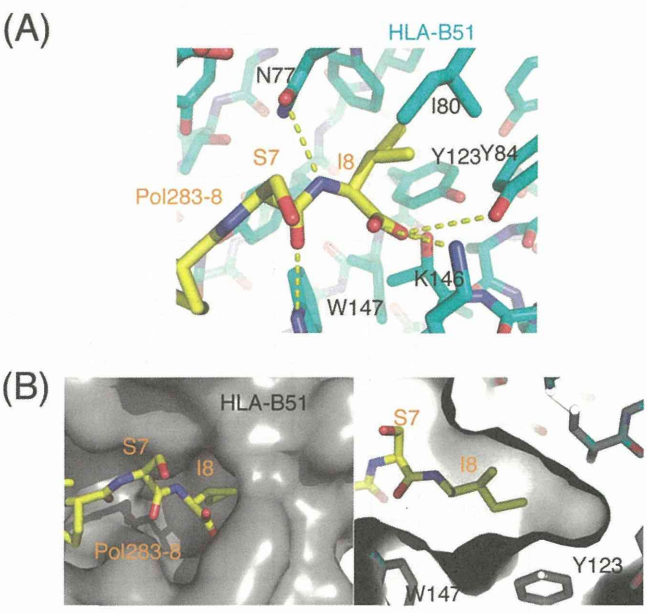

FIG. 6. Binding model of HLA-B*5101 mutant peptides. (A) Polar interactions around the PC residue in the HLA-B51–Pol283-8 complex. The Pol283-8 peptide and the HLA-B51 heavy chain are shown as yellow and cyan stick models, respectively (N and O atoms are shown as blue and red, respectively). The dotted lines indicate hydrogen bonds or salt bridges. (B) (Left) Surface representation (gray) of the HLA-B51 heavy chain with the stick model of the Pol283-8 peptide (with the same coloring as in panel A). 8I (PC) penetrates into the small pocket. (Right) The sliced image of the small PC pocket (right) explains why bulky and long amino acids are not preferential.

HLA-B*57-mediated immune pressure early selects an escape mutant of the TW10 epitope, which has a low viral fitness (29, 32). Escape mutations (K, G, Q, and T at position 242) of the KK10 epitope selected by HLA-B*27-mediated immune pressure impair viral replication, but the compensatory S173A mutation restores viral replication (40, 41). Pol283-8 escape mutations (T, L, and R) are different from those escape mutations, because these Pol283-8 mutations do not influence viral fitness (43). HLA-B*5701 is highly associated with LTNPs, but the mechanism of suppression of HIV-1 replication by epitope-specific CTLs still remains unknown (35, 36). On the other hand, several reports indicate that epitope-specific CTLs in $HLA\text{-}B^*57^+$ LTNPs have the ability to cross-recognize variant epitopes (4, 13, 46), suggesting the control of escape mutants by these CTLs. In the present study, we demonstrated the selection of escape mutations by HLA-B*5101-mediated immune pressure and showed that 2 kinds of mutations, escape mutations for slow progressors and a mutation reducing viral fitness and weakly affecting T-cell recognition for LTNPs, were selected in slow-progressing and LTNP hemophiliacs.

In the present study, we showed that $HLA\text{-}B^*5101^+$ hemo-

philiacs exhibited significantly slow progression during the years 1998 to 2007. Furthermore, we demonstrated that the control of HIV-1 over approximately 25 years in HLA-B*5101-positive hemophiliacs was associated with a Pol283-8-specific $CD8^+$ T-cell response. This is the first study finding that a Pol-specific CTL response is more effective in the control of HIV-1 than a Gag-specific CTL response. Our findings provide a novel mechanism for understanding the long-term control of HIV-1 in LTNPs and slow progressors.

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REFERENCES

1. **Akari, H., S. Arold, T. Fukumori, T. Okazaki, K. Strebel, and A. Adachi.** 2000. Nef-induced major histocompatibility complex class I down-regulation is functionally dissociated from its virion incorporation, enhancement of viral infectivity, and CD4 down-regulation. J. Virol. **74:**2907–2912.
2. **Altman, J. D., P. A. H. Moss, P. J. R. Goulder, D. H. Barouch, M. G. McHeyzer-Williams, J. I. Bell, A. J. McMichael, and M. M. Davis.** 1996. Phenotypic analysis of antigen-specific T lymphocytes. Science **274:**94–96.
3. **Appay, V., D. F. Nixon, S. M. Donahoe, G. M. Gillespie, T. Dong, A. King, G. S. Ogg, H. M. Spiegel, C. Conlon, C. A. Spina, D. V. Havlir, D. D. Richman, A. Waters, P. Easterbrook, A. J. McMichael, and S. L. Rowland-Jones.** 2000. HIV-specific CD8(+) T cells produce antiviral cytokines but are impaired in cytolytic function. J. Exp. Med. **192:**63–75.
4. **Bailey, J. R., T. M. Williams, R. F. Siliciano, and J. N. Blankson.** 2006. Maintenance of viral suppression in HIV-1-infected HLA-B*57+ elite suppressors despite CTL escape mutations. J. Exp. Med. **203:**1357–1369.
5. **Borrow, P., H. Lewicki, B. H. Hahn, G. M. Shaw, and M. B. Oldstone.** 1994. Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. J. Virol. **68:**6103–6110.
6. **Borrow, P., H. Lewicki, X. Wei, M. S. Horwitz, N. Peffer, H. Meyers, J. A. Nelson, J. E. Gairin, B. H. Hahn, M. B. Oldstone, and G. M. Shaw.** 1997. Antiviral pressure exerted by HIV-1-specific cytotoxic T lymphocytes (CTLs) during primary infection demonstrated by rapid selection of CTL escape virus. Nat. Med. **3:**205–211.
7. **Buchbinder, S. P., M. H. Katz, N. A. Hessol, P. M. O'Malley, and S. D. Holmberg.** 1994. Long-term HIV-1 infection without immunologic progression. AIDS **8:**1123–1128.
8. **Collins, K. L., B. K. Chen, S. A. Kalams, B. D. Walker, and D. Baltimore.** 1998. HIV-1 Nef protein protects infected primary cells against killing by cytotoxic T lymphocytes. Nature **391:**397–401.
9. **Crawford, H., W. Lumm, A. Leslie, M. Schaefer, D. Boeras, J. G. Prado, J. Tang, P. Farmer, T. Ndung'u, S. Lakhi, J. Gilmour, P. Goepfert, B. D. Walker, R. Kaslow, J. Mulenga, S. Allen, P. J. R. Goulder, and E. Hunter.** 2009. Evolution of HLA-B*5703 HIV-1 escape mutations in HLA-B*5703-positive individuals and their transmission recipients. J. Exp. Med. **206:**909–921.
10. **Deacon, N. J., A. Tsykin, A. Solomon, K. Smith, M. Ludford-Menting, D. J. Hooker, D. A. McPhee, A. L. Greenway, A. Ellett, C. Chatfield, V. A. Lawson, S. Crowe, A. Maerz, S. Sonza, J. Learmont, J. S. Sullivan, A. Cunningham, D. Dwyer, D. Dowton, and J. Mills.** 1995. Genomic structure of an attenuated quasi species of HIV-1 from a blood transfusion donor and recipients. Science **270:**988–991.
11. **Feeney, M. E., K. A. Roosevelt, Y. Tang, K. J. Pfafferott, K. McIntosh, S. K. Burchett, C. Mao, B. D. Walker, and P. J. R. Goulder.** 2003. Comprehensive screening reveals strong and broadly directed human immunodeficiency virus type 1-specific CD8 responses in perinatally infected children. J. Virol. **77:**7492–7501.
12. **Frahm, N., S. Adams, P. Kiepiela, C. H. Linde, H. S. Hewitt, M. Lichterfeld, K. Sango, N. V. Brown, E. Pae, A. G. Wurcel, M. Altfeld, M. E. Feeney, T. M. Allen, T. Roach, M. A. St. John, E. S. Daar, E. Rosenberg, B. Korber, F. Marincola, B. D. Walker, P. J. R. Goulder, and C. Brander.** 2005. HLA-B63 presents HLA-B57/B58-restricted cytotoxic T-lymphocyte epitopes and is associated with low human immunodeficiency virus load. J. Virol. **79:**10218–10225.
13. **Gillespie, G. M., R. Kaul, T. Dong, H. B. Yang, T. Rostron, J. J. Bwayo, P. Kiama, T. Peto, F. A. Plummer, A. J. McMichael, and S. L. Rowland-Jones.** 2002. Cross-reactive cytotoxic T lymphocytes against a HIV-1 p24 epitope in slow progressors with B*57. AIDS **16:**961–972.
14. **Goulder, P. J., R. E. Philips, R. A. Colbert, S. McAdam, G. Ogg, M. A. Nowak, P. Giangrande, G. Luzzi, B. Morgan, A. Edwards, A. J. McMichael, and S. Rowland-Jones.** 1997. Late escape from an immunodominant cytotoxic T-lymphocyte response associated with progression to AIDS. Nat. Med. **3:**212–217.
15. **Hayashi, H., P. D. Ennis, H. Ariga, R. D. Salter, P. Parham, K. Kano, and M. Takiguchi.** 1989. HLA-B51 and HLA-Bw52 differ only by two amino acids which are in the helical region of α1 domain. J. Immunol. **142:**306–311.
16. **Honeyborne, I., A. Prendergast, F. Pereyra, A. Leslie, H. Crawford, R. Payne, S. Reddy, K. Bishop, E. Moodley, K. Nair, M. van der Stok, N. McCarthy, C. M. Rousseau, M. Addo, J. I. Mullins, C. Brander, P. Kiepiela, B. D. Walker, and P. J. R. Goulder.** 2007. Control of human immunodeficiency virus type 1 is associated with HLA-B*13 and targeting of multiple gag-specific CD8+ T-cell epitopes. J. Virol. **81:**3667–3672.
17. **Huang, Y., L. Zhang, and D. D. Ho.** 1995. Characterization of nef sequences in long-term survivors of human immunodeficiency virus type 1 infection. J. Virol. **69:**93–100.
18. **Itoh, Y., N. Mizuki, T. Shimada, F. Azuma, M. Itakura, K. Kashiwase, E. Kikkawa, J. K. Kulski, M. Satake, and H. Inoko.** 2005. High-throughput DNA typing of HLA-A, -B, -C, and -DRB1 loci by a PCR-SSOP-Luminex method in the Japanese population. Immunogenetics **57:**717–729.
19. **Kaslow, R. A., M. Carrington, R. Apple, L. Park, A. Muñoz, A. J. Saah, J. J. Goedert, C. Winkler, S. J. O'Brien, C. Rinaldo, R. Detels, W. Blattner, J. Phair, H. Erlich, and D. L. Mann.** 1996. Influence of combinations of human major histocompatibility complex genes on the course of HIV-1 infection. Nat. Med. **2:**405–411.
20. **Kawashima, Y., K. Pfafferott, J. Frater, P. Matthews, R. Payne, M. Addo, H. Gatanaga, M. Fujiwara, A. Hachiya, H. Koizumi, N. Kuse, S. Oka, A. Duda, A. Prendergast, H. Crawford, A. Leslie, Z. Brumme, C. Brumme, T. Allen, C. Brander, R. Kaslow, J. Tang, E. Hunter, S. Allen, J. Mulenga, S. Branch, T. Roach, M. John, S. Mallal, A. Ogwu, R. Shapiro, J. G. Prado, S. Fidler, J. Weber, O. G. Pybus, P. Klenerman, T. Ndung'u, R. Phillips, D. Heckerman, P. R. Harrigan, B. D. Walker, M. Takiguchi, and P. Goulder.** 2009. Adaptation of HIV-1 to human leukocyte antigen class I. Nature **458:**641–645.
21. **Keet, I. P., A. Krol, M. R. Klein, P. Veugelers, J. de Wit, M. Roos, M. Koot, J. Goudsmit, F. Miedema, and R. A. Coutinho.** 1994. Characteristics of long-term asymptomatic infection with human immunodeficiency virus type 1 in men with normal and low CD4+ cell counts. J. Infect. Dis. **169:**1236–1243.
22. **Kiepiela, P., A. J. Leslie, I. Honeyborne, D. Ramduth, C. Thobakgale, S. Chetty, P. Rathnavalu, C. Moore, K. J. Pfafferott, L. Hilton, P. Zimbwa, S. Moore, T. Allen, C. Brander, M. M. Addo, M. Altfeld, I. James, S. Mallal, M. Bunce, L. D. Barber, J. Szinger, C. Day, P. Klenerman, J. Mullins, B. Korber, H. M. Coovadia, B. D. Walker, and P. J. Goulder.** 2004. Dominant influence of HLA-B in mediating the potential co-evolution of HIV and HLA. Nature **432:**769–775.
23. **Kiepiela, P., K. Ngumbela, C. Thobakgale, D. Ramduth, I. Honeyborne, E. Moodley, S. Reddy, C. de Pierres, Z. Mncube, N. Mkhwanazi, K. Bishop, M. van der Stok, K. Nair, N. Khan, H. Crawford, R. Payne, A. Leslie, J. Prado, A. Prendergast, J. Frater, N. McCarthy, C. Brander, G. H. Learn, D. Nickle, C. Rousseau, H. Coovadia, J. I. Mullins, D. Heckerman, B. D. Walker, and P. Goulder.** 2007. CD8+ T-cell responses to different HIV proteins have discordant associations with viral load. Nat. Med. **13:**46–53.
24. **Kirchhoff, F., T. C. Greenough, D. B. Brettler, J. L. Sullivan, and R. C. Desrosiers.** 1995. Absence of intact nef sequences in a long-term survivor with nonprogressive HIV-1 infection. N. Engl. J. Med. **332:**228–232.
25. **Klein, M. R., C. A. van Baalen, A. M. Holwerda, S. R. K. Garde, R. J. Bende, I. P. Keet, J. K. Eeftinck-Schattenkerk, A. D. Osterhaus, H. Schuitemaker, and F. Miedema.** 1995. Kinetics of Gag-specific cytotoxic T lymphocyte responses during the clinical course of HIV-1 infection: a longitudinal analysis of rapid progressors and long-term asymptomatics. J. Exp. Med. **181:**1365–1372.
26. **Koup, R. A., J. T. Safrit, Y. Cao, C. A. Andrews, G. McLeod, W. Borkowsky, C. Farthing, and D. D. Ho.** 1994. Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. J. Virol. **68:**4650–4655.
27. **Kwong, P. D., M. L. Doyle, D. J. Casper, C. Cicala, S. A. Leavitt, S. Majeed, T. D. Steenbeke, M. Venturi, I. Chaiken, M. Fung, H. Katinger, P. W. Parren, J. Robinson, D. Van Ryk, L. Wang, D. R. Burton, E. Freire, R. Wyatt, J. Sodroski, W. A. Hendrickson, and J. Arthos.** 2002. HIV-1 evades antibody-mediated neutralization through conformational masking of receptor-binding sites. Nature **420:**678–682.
28. **Leslie, A., D. Kavanagh, I. Honeyborne, K. Pfafferott, C. Edwards, T. Pillay, L. Hilton, C. Thobakgale, D. Ramduth, R. Draenert, S. Le Gall, G. Luzzi, A. Edwards, C. Brander, A. K. Sewell, S. Moore, J. Mullins, C. Moore, S. Mallal, N. Bhardwaj, K. Yusim, R. Phillips, P. Klenerman, B. Korber, P.**

Kiepiela, B. Walker, and P. Goulder. 2005. Transmission and accumulation of CTL escape variants drive negative associations between HIV polymorphisms and HLA. J. Exp. Med. **201:**891–902.

29. **Leslie, A. J., K. J. Pfafferott, P. Chetty, R. Draenert, M. M. Addo, M. Feeney, Y. Tang, E. C. Holmes, T. Allen, J. G. Prado, M. Altfeld, C. Brander, C. Dixon, D. Ramduth, P. Jeena, S. A. Thomas, A. St John, T. A. Roach, B. Kupfer, G. Luzzi, A. Edwards, G. Taylor, H. Lyall, G. Tudor-Williams, V. Novelli, J. Martinez-Picado, P. Kiepiela, B. D. Walker, and P. J. Goulder.** 2004. HIV evolution: CTL escape mutation and reversion after transmission. Nat. Med. **10:**282–289.
30. **Maenaka, K., T. Maenaka, H. Tomiyama, M. Takiguchi, D. I. Stuart, and E. Y. Jones.** 2000. Nonstandard peptide binding revealed by crystal structures of HLA-B*5101 complexed with HIV immunodominant epitopes. J. Immunol. **165:**3260–3267.
31. **Magierowska, M., I. Theodorou, P. Debré, F. Sanson, B. Autran, Y. Riviere, D. Charron, and D. Costagliola.** 1999. Combined genotypes of CCR5, CCR2, SDF1, and HLA genes can predict the long-term nonprogressor status in human immunodeficiency virus-1-infected individuals. Blood **93:** 936–941.
32. **Martinez-Picado, J., J. G. Prado, E. E. Fry, K. Pfafferott, A. Leslie, S. Chetty, C. Thobakgale, I. Honeyborne, H. Crawford, P. Matthews, T. Pillay, C. Rousseau, J. I. Mullins, C. Brander, B. D. Walker, D. I. Stuart, P. Kiepiela, and P. Goulder.** 2006. Fitness cost of escape mutations in p24 Gag in association with control of human immunodeficiency virus type 1. J. Virol. **80:**3617–3623.
33. **Matsumoto, K., J. Yamamoto, M. Hiraiwa, K. Kano, and M. Takiguchi.** 1990. Discrimination of HLA-B5 cross reactive group antigen by human allospecific CTL clones. Transplantation **49:**1164–1167.
34. **Matthews, P. C., A. Prendergast, A. Leslie, H. Crawford, R. Payne, C. Rousseau, M. Rolland, I. Honeyborne, J. Carlson, C. Kadie, C. Brander, K. Bishop, N. Mlotshwa, J. I. Mullins, H. Coovadia, T. Ndung'u, B. D. Walker, D. Heckerman, and P. J. R. Goulder.** 2008. Central role of reverting mutations in HLA associations with human immunodeficiency virus set point. J. Virol. **82:**8548–8559.
35. **Migueles, S. A., A. C. Laborico, H. Imamichi, W. L. Shupert, C. Royce, M. McLaughlin, L. Ehler, J. Metcalf, S. Liu, C. W. Hallahan, and M. Connors.** 2003. The differential ability of HLA B*5701+ long-term nonprogressors and progressors to restrict human immunodeficiency virus replication is not caused by loss of recognition of autologous viral gag sequences. J. Virol. **77:**6889–6898.
36. **Migueles, S. A., M. S. Sabbaghian, W. L. Shupert, M. P. Bettinotti, F. M. Marincola, L. Martino, C. W. Hallahan, S. M. Selig, D. Schwartz, J. Sullivan, and M. Connors.** 2000. HLA B*5701 is highly associated with restriction of virus replication in a subgroup of HIV-infected long term nonprogressors. Proc. Natl. Acad. Sci. U. S. A. **97:**2709–2714.
37. **O'Brien, S. J., X. Gao, and M. Carrington.** 2001. HLA and AIDS: a cautionary tale. Trends Mol. Med. **7:**379–381.
38. **Pantaleo, G., S. Menzo, M. Vaccarezza, C. Graziosi, O. J. Cohen, J. F. Demarest, D. Montefiori, J. M. Orenstein, C. Fox, L. K. Schrager, J. B. Margolick, S. Buchbinder, J. V. Giorgi, and A. S. Fauci.** 1995. Studies in subjects with long-term nonprogressive human immunodeficiency virus infection. N. Engl. J. Med. **332:**209–216.
39. **Sacha, J. B., C. Chung, J. Reed, A. K. Jonas, A. T. Bean, S. P. Spencer, W. Lee, L. Vojnov, R. Rudersdorf, T. C. Friedrich, N. A. Wilson, J. D. Lifson, and D. I. Watkins.** 2007. Pol-specific CD8+ T cells recognize simian immunodeficiency virus-infected cells prior to Nef-mediated major histocompatibility complex class I downregulation. J. Virol. **81:**11703–11712.
40. **Schneidewind, A., M. A. Brockman, J. Sidney, Y. E. Wang, H. Chen, T. J. Suscovich, B. Li, R. I. Adam, R. L. Allgaier, B. R. Mothé, T. Kuntzen, C. Oniangue-Ndza, A. Trocha, X. G. Yu, C. Brander, A. Sette, B. D. Walker, and T. M. Allen.** 2008. Structural and functional constraints limit options for cytotoxic T-lymphocyte escape in the immunodominant HLA-B27-restricted epitope in human immunodeficiency virus type 1 capsid. J. Virol. **82:**5594–5605.
41. **Schneidewind, A., M. A. Brockman, R. Yang, R. I. Adam, B. Li, S. L. Gall, C. R. Rinaldo, S. L. Craggs, R. L. Allgaier, K. A. Power, T. Kuntzen, C. S. Tung, M. X. LaBute, S. M. Mueller, T. Harrer, A. J. McMichael, P. J. R. Goulder, C. Aiken, C. Brander, A. D. Kelleher, and T. M. Allen.** 2007. Escape from the dominant HLA-B27-restricted cytotoxic T-lymphocyte response in Gag is associated with a dramatic reduction in human immunodeficiency virus type 1 replication. J. Virol. **81:**12382–12393.
42. **Tomiyama, H., H. Akari, A. Adachi, and M. Takiguchi.** 2002. Different effects of Nef-mediated HLA class I down-regulation on HIV-1-specific CD8+ T cell cytokine activity and cytokine production. J. Virol. **76:**7535–7543.
43. **Tomiyama, H., M. Fujiwara, S. Oka, and M. Takiguchi.** 2005. Epitope-dependent effect of Nef-mediated HLA class I down-regulation on ability of HIV-1-specific CTLs to suppress HIV-1 replication. J. Immunol. **174:**36–40.
44. **Tomiyama, H., N. Yamada, H. Komatsu, K. Hirayama, and M. Takiguchi.** 2000. A single CTL clone can recognize a naturally processed HIV-1 epitope presented by two different HLA class I molecules. Eur. J. Immunol. **30:**2521–2530.
45. **Tomiyama, H., T. Sakaguchi, K. Miwa, S. Oka, A. Iwamoto, Y. Kaneko, and M. Takiguchi.** 1999. Identification of multiple HIV-1 CTL epitopes presented by HLA-B*5101 molecules. Hum. Immunol. **60:**177–186.
46. **Turnbull, E. L., A. R. Lopes, N. A. Jones, D. Cornforth, P. Newton, D. Aldam, P. Pellegrino, J. Turner, I. Williams, C. M. Wilson, P. A. Goepfert, M. K. Maini, and P. Borrow.** 2006. HIV-1 epitope-specific CD8+ T cell responses strongly associated with delayed disease progression cross-recognize epitope variants efficiently. J. Immunol. **176:**6130–6146.
47. **Wei, X., J. M. Decker, S. Wang, H. Hui, J. C. Kappes, X. Wu, J. F. Salazar-Gonzalez, M. G. Salazar, J. M. Kilby, M. S. Saag, N. L. Komarova, M. A. Nowak, B. H. Hahn, P. D. Kwong, and G. M. Shaw.** 2003. Antibody neutralization and escape by HIV-1. Nature **422:**307–312.
48. **Wyatt, R., and J. Sodroski.** 1998. The HIV-1 envelope glycoproteins: fusogens, antigens, and immunogens. Science **280:**1884–1888.
49. **Zuñiga, R., A. Lucchetti, P. Galvan, S. Sanchez, C. Sanchez, A. Hernandez, H. Sanchez, N. Frahm, C. H. Linde, H. S. Hewitt, W. Hildebrand, M. Altfeld, T. M. Allen, B. D. Walker, B. T. Korber, T. Leitner, J. Sanchez, and C. Brander.** 2006. Relative dominance of Gag p24-specific cytotoxic T lymphocytes is associated with human immunodeficiency virus control. J. Virol. **80:**3122–3125.

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Original article

Impact of CRF01_AE-specific polymorphic mutations G335D and A371V in the connection subdomain of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) on susceptibility to nucleoside RT inhibitors

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Abstract

Certain mutations in the connection subdomain and RNase H domain of reverse transcriptase (RT) of subtype B HIV-1 contribute to resistance to nucleoside reverse transcriptase inhibitors (NRTIs). However, the impact of non-B subtype polymorphisms in this region on drug resistance remains unclear. In this study, we determined the frequencies of drug resistance mutations of the entire RT in patients with treatment failure from a cohort of Circulating recombinant form (CRF) 01_AE HIV-1-infected patients in Hanoi, Viet Nam. Subsequently, we assessed the impact of CRF01_AE polymorphisms G335D and A371V with or without thymidine analogue mutations (TAMs) on susceptibility to NRTI with recombinant viruses. In 49 patients with treatment failure, resistance mutations to NRTIs in the N-terminal half of RT were observed in 89.8%. In the C-terminal half, G335D (100%), N348I (36.8%), A371V (100%), A376S (5.3%) and A400T (97.4%) were detected, although G335D, A371V and A400T were considered polymorphisms of CRF01_AE. Drug susceptibility showed G335D, A371V, or both did not confer resistance by themselves but conferred significant resistance to NRTIs with TAMs, especially in mutants containing G335D, A371V and TAM type 2. Our results suggest the important role of CRF01_AE polymorphisms in the C-terminal half of RT in drug resistance.

Keywords: Drug resistance; Reverse transcriptase; G335D; A371V; CRF01_AE

1. Introduction

In Viet Nam, where the epidemic of human immune deficiency virus type 1 (HIV-1) has been in a rapid growth phase with an estimated number of HIV-1-infected individuals rising from 122 × 10^3 in 2000 to 283 × 10^3 in 2006, the intensive introduction of antiretroviral therapy (ART) has been implemented with two nucleoside reverse transcriptase inhibitors (NRTI) and one non-nucleoside reverse transcriptase inhibitor (NNRTI) [1,2] and ART coverage of HIV-1-infected individuals has increased from 1% in 2003 to 28.4% in 2007 [3–5]. At the same time, concern regarding drug resistance has emerged [6].

HIV-1 reverse transcriptase (RT) is a heterodimer of two subunits: a 66-kDa subunit (p66) and a 51-kDa subunit and the p66 contains the N-terminal polymerase (codons 1–321), the connection subdomain (codons 322–440) and RNase H (codons 441–560). Although the majority of commercially available genotypic and phenotypic assays have not targeted

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the C-terminal half of RT: the connection subdomain and RNase H domains, certain mutations in this region have been recently found to be associated with resistance to NRTIs and NNRTIs [7–20]. Despite the accumulation of data on the prevalence and mechanisms of mutations in the C-terminal half of RT, most data are from subtype B viruses and little information is available on those of non-B subtype. Since amino acid sequence diversity in the *pol* gene is 10–15% among subtypes [21–23] and the subtype have an impact on drug resistance mutations [19–31], there is a need to determine whether inter-subtype diversity influences the spectrum of drug resistance mutations in the C-terminal half of RT as well.

Circulating recombinant form (CRF) 01_AE is the most predominant subtype in Viet Nam [32–34] and accounting for 83% of all HIV-1 infections in Southeast Asia [28–34]. Recently, Delviks-Frankenberry demonstrated that the substitution A400T, a common polymorphism in RNase H of CRF01_AE, is responsible for the high AZT resistance [16], although A400T usually emerged after AZT exposure in subtype B [19]. As well as A400T, we found a high frequency of G335D and A371V in treatment-naïve CRF01_AE patients [20]. Although G335D and A371V are assumed as common polymorphisms in CRF01_AE in the Stanford HIV Drug Resistance Database [http://hivdb.stanford.edu/index.html, accessed as late as July 20th 2010], they are thought to be associated with AZT resistance in subtype B [7,11]. However, the role of substitutions G335D and A371V in drug resistance to NRTIs has not been well characterized.

In the present study, we first investigated drug resistance mutations of CRF01_AE HIV-1 including the connection subdomain and RNase H domain of RT from HIV-1-infected patients failing ART. In addition, since we again found high frequencies of the double mutation of G335D and A371V in this population, we examined phenotypic resistance levels of these mutations by using mutant recombinant viruses containing G335D, A371V or both with or without TAMs, to determine the impacts of these mutations on drug resistance.

2. Materials and methods

2.1. Study population

HIV-1-infected patients who had taken antiretroviral therapy for more than 6 months at the National Hospital for Tropical Diseases (NHTD) in Hanoi between October 1, 2007 and June 30, 2008, were enrolled in this study. Each participant provided a written informed consent. Plasma viral load (pVL) was measured by the Cobas AmpliPrep-Cobas TaqMan system (Roche Diagnostics, Tokyo, Japan) and plasma samples were stored at −80 °C for genotypic resistance testing. When pVL was >1000 copies/ml, the patient was defined as treatment failure and the frozen plasma was shipped to the National Center for Global Health and Medicine (NCGM) in Tokyo for genotypic resistance testing.

The study protocol was approved by the institutional ethical review boards of NTHD and NCGM (IMCJ-H18-360) and by the ethics committee of the Vietnamese Ministry of Health (#1468,1469/QD-BYT dated April 19, 2007).

2.2. Reagents and cells

AZT, stavudine (d4T) and didanosine (ddI) were purchased from Sigma (St. Louis, MO). Lamivudine (3 TC) and tenofovir (TDF) were purchased from Moravek Biochemicals, Inc. (Brea, CA). Abacavir (ABC) was generously provided by GlaxoSmithKline (Philadelphia, PA). Cos-7 and MAGIC-5 cells (CCR5-transduced HeLa-CD4/LTR-β-Gal cells) were cultured and used as described previously [35].

2.3. Genotypic resistance and subtype analysis

Drug resistance genotyping was carried out by in-house protocols in NCGM. In brief, total RNA was extracted from plasma with a High Pure Viral RNA kit (Boehringer Mannheim, Mannheim, Germany), followed by reverse transcription-polymerase chain reaction (PCR) with a One Step RNA PCR kit (TaKaRa Shuzo, Otsu, Japan). Nested PCR was subsequently conducted with a Prime STAR Max Premix kit (TaKaRa Shuzo, Otsu, Japan) to amplify nearly the entire RT region (codons 1–560) and protease region. The primer sets for amplification of the N-terminal half of RT (codons 1–318) were T1-AE [5′-AGGGGGAATTGGAGGTTT; nucleotides (nt) 2393–2410] and T4-AE (5′-TTCTGTTAGTGCTTTGGTT; nt 3422–3404) for the first PCR, and T12-AE (5′-CCAGTAAAATTAAAGCCAG; nt 2574–2592) and T15-AE (5′-TCCCACTAACTTCTGTATGTC; nt 3335–3315) for the second PCR. The primer sets for amplification of the C-terminal half of RT (codons 319–560) were 3120F-AE (5′-TCTGATTTAGAAATAGGGCAG; nt 3120–3140) and 4428R-AE (5′-GTGTGC AATCTAATTGCCATAT; nt 4428–4407) for the first PCR, and 3240F-AE (5′-GGATATGAACTCCATCCTGA; nt 3240–3259) and 4316R-AE (5′-GTGGCAAATTAAAATCACTAGCC; nt 4316–4295) for the second PCR. Primer sets for amplification of protease were PR01-AE (5′-CCAACAGCCCCACCAGC; nt 2152–2168) and PR02AE (5′-ATTTTCAGGCCCAATT TTTGA; nt 2711–2691) for the first PCR, and PR03-AE (5′-AGCAGGAGCAGAAAGACAAGG; nt 2213 to) and PR04-AE (5′-CTGGCTTTAATKTTACTGGTA; nt 2592–2572) for the second PCR. The PCR products were purified with QIAquick PCR Purification Kit (Qiagen, Valencia, CA) and subjected to direct sequencing with an ABI PRISM 3730 automated DNA sequencer (Applied Biosystems, Foster City, CA). Amino acid sequences were deduced with the Genetyx-Win program version 8.0 (Software Development, Tokyo).

Resistance-associated mutations in the N-terminal half of RT were identified according to the International AIDS Society Resistance-USA Panel revised in December 2009 [36] and subtypes of HIV-1 in RT gene were determined by software "Genotyping/NCBI" using BLAST algorithm [http://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi]. Resistant mutations in the connection subdomain and the RNase H domain of RT in the previous reports were determined if greater than three-fold increase of EC_{50} compared to

that of NL4-3 was noted in the reports. Since all sequences of the study participants belonged to CRF01_AE subtype, data on frequencies of each mutation in the C-terminal half of RT in CRF01_AE and subtype B in treatment-naïve patients was obtained from the Stanford HIV Drug Resistance Database [http://hicdb.stanford.edu/index.html, accessed as late as July 20th, 2010] for reference. Nucleotide sequences of the C-terminal half of CRF01_AE RT from 38 patients have been deposited in the DDBJ database (accession numbers AB545813–AB545850).

2.4. Construction of recombinant HIV-1 harboring G335D and/or A371V with or without TAMs

To examine the influence of G335D and A371V on drug susceptibility to NRTIs, we constructed mutant HIV-1 recombinants that included G335D, A371V or both with or without TAMs. TAM-1 virus was constructed as combination of M41L, L210W and T215Y and TAM-2 as combination of D67N, K70R and T215F. Mutant recombinant plasmid clones of the virus were generated by oligonucleotide site-directed mutagenesis as described previously [10], using pBS-RT_{WT}, which contains the entire RT coding sequences (amino acid position 14–560) and three silent restriction sites (XmaI, NheI and XbaI from the 5′ to 3′ end of RT at codons 15, 267, and 560). After site-directed mutagenesis, the mutated RT was ligated into pNL4-3, which contains the entire genome of HIV-1 and the same silent restriction sites as pBS-RT_{WT}. The infectious virus was generated by transfection of each molecular clone into Cos-7 cells, harvested and stored at −80 °C until use. Infectivity was measured as blue cell-forming units (BFU) of MAGIC-5 cells. All mutations in recombinant viruses were confirmed by full-length sequencing of the entire RT coding region.

2.5. Drug susceptibility assay

Susceptibility to NRTIs was determined by using MAGIC-5 cells as described previously [35] in more than three experiments. MAGIC-5 cells were infected with diluted virus stock (100 BFU) in the presence of increasing concentrations of RTIs, cultured for 48 h, fixed and stained with X-Gal (5-bromo-4chloro-3-indolyl-βD-galactopyranoside). The stained cells were counted under a light microscope. Drug concentrations that reduced the cell count to 50% of that of the drug-free control (EC_{50}) were determined by referring to the dose–response curve.

2.6. Statistical analysis

Data are expressed as mean ± SD. The Student's t-test was used to compare two groups of continuous variables and a p-value less than 0.05 was considered statistically significant. Statistical analyses were performed using SPSSII software package for Windows, version 11.0J (SPSS Japan Inc, Tokyo, Japan).

3. Results

3.1. Characteristics of patients failing antiretroviral therapy

A total of 416 individuals on ART were consecutively enrolled in the present study and their pVLs were assayed between October 1, 2007 and June 30, 2008 at the NTHD in Hanoi. Among them, 49 individuals were confirmed as treatment failure by the definition described above and assigned for genotypic resistance analysis. The characteristics of the 49 individuals are listed in Table 1. All patients had received AZT or d4T plus 3TC combined with NVP, EFV or lopinavir/ritonavir (LPV/r) at the time of enrollment. The most frequently used combination was AZT, 3TC, and NVP, followed by d4T, 3TC and NVP. Protease inhibitors (PIs) were used by 17 (34.7%) patients, while the Vietnamese national ART guideline recommends d4T, 3TC plus 1 NNRTI for the first line regimen [2]. This was probably due to the inclusion of patients who had started ART when the guideline had not been issued yet. The median duration of ART exposure was 2.98 years (IQR 2.17–4.00).

3.2. Genotypic resistance patterns including C-terminal domain of RT

We successfully amplified the N-terminal half of RT and protease of all the 49 patients and C-terminal region of RT of 38 patients. The proportion of patients with at least one NRTI resistance mutation in the N-terminal half of RT was 89.8%.

Table 1
Characteristics of patients failing antiretroviral therapy.

		$n = 49$	(%)
Sex, n (%)			
males		33	(67.3)
females		16	(32.7)
Median Age, years (range)		31	(21–50)
Risk of HIV-1 infection (multiple choice), n (%)			
sexual contact		46	(93.9)
intravascular drug use		20	(40.8)
CD4 count, median cells/mm^3 (IQR)		145	(84–195)
Plasma viral load, median log copies/ml (IQR),		4.23	(3.59–4.94)
Duration of prior ART, median years (IQR)		2.98	(2.17–4.00)
Experienced ART, n (%)			
NRTI	AZT	39	(79.6)
	d4T	24	(49.0)
	3TC	49	(100)
	ddI	7	(14.3)
	ABC	2	(4.1)
	TDF	2	(4.1)
NNRTI	NVP	43	(87.8)
	EFV	15	(30.6)
PI	IDV	12	(24.5)
	SQV	6	(12.2)
	LPVr	3	(6.1)

IQR: interquartile range. ART: antiretroviral therapy. NRTI: nucleoside reverse transcriptase inhibitor. NNRTI: non-nucleoside reverse transcriptase inhibitor. PI: protease inhibitor.

Of those, M184V was the most common (81.6%) and TAMs were also observed frequently in 71.4%: M41L (22.4%), D67N (24.5%), K70R (18.4%), L210W (14.3%), T215F (16.3%), T215Y (28.6%), K219E (12.2%) and K219Q (6.1%), whereas K65R (6.1%), L74V (4.1%), Y115F (2.0%) and mutations driven by Q151M complex (4.1%) were relatively rare. Similar to previous reports on drug resistance in CRF01_AE [28–30], mutations classified into TAM type 2 (TAM-2): D67N, K70R, T215F and K219E/Q, were more frequently observed than those of TAM type 1 (TAM-1): M41L, L210W and T215Y/F (30.6% v.s. 26.5%), except for a patient having only T215F. With regard to codon 215, T215F were more frequently seen with other TAM-2 mutations (six out of eight sequences that contain T215F), concurring with the previous reports showing the introduction of T215F into TAM-2 backbone increase relative fitness in the presence of AZT but resulted in decreased viral fitness in TAM-1 backbone [37]. The resistance mutations of NNRTIs in the N-terminal half of RT were detected in 79.6%. The most frequent NNRTI-resistance mutations were Y181C/I/V (32.7%), K103N (26.5%) and G190A (26.5%). In 17 PI experienced patients, no major mutations were found, but 9 minor mutations were detected: L10I/V (11.8%), I13V (88.2%), G16E (11.8%), K20R (17.6%), M36I (100%), L63P (29.4%), H69K (100%), V82I (11.8%) and I93L (8.2%). However, the mutations in protease are considered as consensus amino acids in most non-B subtype HIV-1 (I13V, M36I and H69K) or common polymorphic mutations (L10V, G16E, K20R, L63P, V82I and I93L) and could not be determined as mutations that emerged after treatment.

The frequencies of mutations in the C-terminal half of the RT reported previously as NRTI or NNRTI resistance [7–20] are described in Table 2. As shown, G335D (100%), N348I (36.8%), A371V (100%), A376S (5.3%), E399D (28.9%) and A400T (97.4%) were detected in the patients failing ART. However, as we reported previously [20], G335D and A371V were also commonly observed in untreated patients infected with non-B subtype HIV-1 and the frequencies of G335D and A371V in CRF01_AE subtype shown in the Stanford HIV Drug Resistance Database are 95.2% and 97.1%, respectively, while those are rare in subtype B (G335D: 1.3%, A371V: 3.2%). A400T is also one of the known polymorphisms in CRF01_AE [16]. Therefore, it is unlikely that G335D, A371V and A400T in this population were selected by ART exposure or involved in the resistance mutations.

Table 2
Frequencies of mutations associated with RTI-resistance in the connection and RNase H domain of reverse transcriptase of HIV-1.

Mutations[b]	Study participants (Treatment failure)		Stanford database[a] (RTI-naïve)	
	CRF01_AE		CRF01_AE	Subtype B
	$n = 38$			
	%	(n)	%	%
G333	100	(38)		
D	0	(0)	0	0.7
E	0	(0)	0	7.5
G335	0	(0)		
C	0	(0)	0	0.5
D	100	(38)	92.0	1.3
N348	57.9	(22)		
I	36.8	(14)	0	0.5
T	5.3	(2)	0	0
A360	97.4	(37)		
I	0	(0)	0	0
V	0	(0)	0	0.7
S	2.6	(1)	1.1	0
V365	100	(38)		
I	0	(0)	0	3.2
T369	94.7	(36)		
I	0	(0)	0	0
A	2.6	(1)	19.3	3.3
V	2.6	(1)	2.8	1.2
A371	0	(0)		
V	100	(38)	97.1	3.2
A376	94.7	(36)		
S	5.3	(2)	1.7	5.8
E399	*68.4*	*(26)*		
D	*28.9*	*(11)*	*2.6*	*14*
K	*2.6*	*(1)*	*0*	*0.1*
A400	0	(0)		
T	97.4	(37)	89.2	25.3
L	2.6	(1)	0	1
Q475	100	(38)		
A	0	(0)	0	0
Q509	97.4	(37)		
L	0	(0)	0	0
R	2.6	(1)	0	0

[a] Available from http://hicdb.stanford.edu/index.html.
[b] Resistance mutations reported previously [8–21] are indicated in bold. Resistance was defined as greater than three fold increase of EC_{50} compared to that of NL4-3.

3.3. Drug susceptibility assay for mutant recombinant HIV-1

To address whether G335D or A371V have an impact on NRTI susceptibility depending on the pattern of TAMs, we constructed recombinant viruses containing G335D and/or A371V in the background of TAM-1 or TAM-2 by site-directed mutagenesis. As shown in Table 3, G335D, A371V or their double mutant did not increase the resistance levels to all NRTIs by themselves. In contrast, as shown in Table 4, variants with G335D, A371V or both exhibited higher resistance to AZT in the background of TAM-1 (8.2- to 23.2-fold) and the increased resistance level was the greatest in the double mutant G335D/A371V. Although G335D/A371V showed statistical increase in resistance to all the other NRTIs except 3TC, the fold increase from TAM-1 mutant was the greatest in AZT (Table 4). Similar to TAM-1 background, G335D, A371V or G335D/A371V with TAM-2 exhibited considerable increase in susceptibility to AZT (52.7-, 21.1-, 52.6-fold, respectively). In addition, there were marginal changes in d4T susceptibility (Table 5) in the three patterns of the mutants, G335D, A371V or G335D/A371V. In TAM-2 background, we also found G335D alone increased susceptibility to ABC (4.2-fold) and to TDF (2.4-fold), and that G335D/A371V increased susceptibility to ddI (7.2-fold), ABC (3.1-fold) and

Table 3
Drug susceptibilities of HIV-1 variants with G335D or A371V.

Mutation[a]	EC_{50} (μM)[b] (fold increase)											
	AZT		d4T		ddI		3TC		ABC		TDF	
Wild Type	0.050 ± 0.002		2.55 ± 0.07		1.90 ± 0.17		0.45 ± 0.035		2.48 ± 0.21		0.020 ± 0.0023	
335D	0.052 ± 0.004	(1)	3.19 ± 0.14	(1.3)	4.56 ± 0.20	(2.4)	0.45 ± 0.022	(1)	2.71 ± 0.17	(1.1)	0.018 ± 0.0019	(0.9)
371V	0.047 ± 0.003	(0.9)	3.26 ± 0.17	(1.3)	5.30 ± 0.02	(2.8)	0.55 ± 0.027	(1.2)	2.32 ± 0.09	(0.9)	0.027 ± 0.0014	(1.3)
335D/371V	0.052 ± 0.010	(1)	3.52 ± 0.06	(1.4)	3.38 ± 0.21	(1.8)	0.65 ± 0.023	(1.5)	2.39 ± 0.12	(1)	0.025 ± 0.0031	(1.2)

AZT, zidovudine; d4T, stavudine; ddI, didanosine; 3TC, lamivudine; ABC, abacavir; TDF, tenofovir.
[b] Data are mean ± SD from at least three independent experiments. Fold increase was the relative change in EC_{50} value compared with that of HIV-1 WT.
[a] See Materials and Methods for the construction of clones.

TDF (5.2-fold). Of note, the increased resistance levels to AZT, d4T, ddI and TDF were greater in G335D/A371V in TAM-2 background than that in TAM-1 background. Our data suggest double mutant G335D/A371V in TAM-2 background would have the most impact on NRTI susceptibility.

4. Discussion

In the present study, we described the drug resistance mutations in the entire RT of CRF01_AE HIV-1-infected Vietnamese patients who had high pVL levels despite 6-month ART. According to the criteria used for evaluation of drug resistance proposed by Shafer et al. [38,39], correlations between mutations and treatment should be confirmed by extensive resistance surveillance. However, limited sequences of CRF01_AE in the connection subdomain and RNase H domain of the RT have been available so far especially from treatment-experienced patients [40]. Santos et al. [19] previously compared amino acid variations between treatment-naïve and treatment-experienced patients in connection subdomain (280 naïve vs. 230 treated) and RNase H domain (334 naïve vs. 234 treated). Although their study included substantial number of patients, larger number of cases belonged to subtype B (80–82% of treatment-experienced patients) and the unique characteristics of CRF01_AE, accounting for only 10% of their study, could not be fully assessed. Since our present study focused on CRF01_AE sequence alone, the data provide direct information on the evaluation of drug resistance mutations in CRF01_AE, although sequences before ART initiation were not available. The largest study to date exploring treatment-related mutation in RT C-terminal site in CRF01_AE infection is the report from Thailand by Saeng-aroon et al. [40], in which significantly higher frequencies of N348I, E399D, P537S and I542M in treatment-exposed patients than treatment-naïve patients (76 naïve vs. 49 treated) was noted. Although the former two mutations have already known to be associated with exposure to NRTI or NNRTI and were detected in our treatment-experienced patients, the results of P537S and I542M were different from us: no patients in our study had P537S and I542M. Further studies are required to determine the prevalence of drug resistance mutations in the C-terminal half of RT in CRF01_AE.

Among the mutations previously reported as drug resistance in the connection subdomain and RNase H domain of RT, we found no mutations except G335D, N348I, A371V, A376S, E399D and A400T in treatment-experienced individuals with CRF01_AE infection. Of these mutations, N348I is one of the most extensively assessed mutations in the RT connection domain and has been established as multiclass resistance to both NRTIs and NNRTIs by being identified in clinical isolates in treatment-experienced individuals in subtype B and by *in vitro* drug susceptibility assay [9,10,12,13]. Since N348I is rare in treatment-naïve of both subtype B and CRF01_AE, N348I observed in 35.8% of CRF01_AE sequences in our study was considered to be treatment-related. The wide use of NVP in Viet Nam might be one of the causes of the higher prevalence of N348I in this population than in subtype B. In addition to N348I, E399D has been thought to be associated with resistance to AZT and to EFV when combined with K103R and 179D [41,42]. Although our results of E399D prevalence of in treatment-exposed patients (28.9%) was relatively higher than those in the Stanford database (9%), it was similar to the previous study by Saeng-aroon et al. of treatment-exposed patients with CRF01_AE infection (32.7%) and considered to be selected after treatment. In contrast, A376S detected in this study was not clearly identified as a treatment-related mutation because the frequency (5.3%) was similar to those of treatment-naïve

Table 4
Drug susceptibilities of HIV-1 variants with G335D or A371V in the TAM-1 background.

Mutation	EC_{50} (μM) (fold change)											
	AZT		d4T		ddI		3TC		ABC		TDF	
Wild Type	0.050 ± 0.002		2.55 ± 0.07		1.90 ± 0.17		0.45 ± 0.035		2.48 ± 0.21		0.020 ± 0.0023	
TAM-1	0.200 ± 0.016	**(4)**	4.78 ± 0.30	(1.9)	5.35 ± 0.79	(2.8)	2.37 ± 0.017	**(5.3)**	4.20 ± 0.25	(1.7)	0.043 ± 0.0030	(2.2)
TAM-1/335D	0.411 ± 0.028	**(8.2)**[a]	6.63 ± 0.05	(2.6)	5.71 ± 0.57	(3.0)	2.14 ± 0.099	**(4.8)**	3.17 ± 0.23	(1.3)	0.024 ± 0.0026	(1.2)
TAM-1/371V	0.473 ± 0.052	**(9.4)**[a]	6.07 ± 0.12	(2.4)	6.30 ± 0.48	**(3.3)**	2.45 ± 0.110	**(5.5)**	3.88 ± 0.32	(1.6)	0.046 ± 0.0018	(2.3)
TAM-1/335D/371V	1.160 ± 0.078	**(23.2)**[a]	9.01 ± 0.20	**(3.5)**[a]	7.87 ± 0.35	**(4.1)**[a]	2.40 ± 0.016	**(5.4)**	7.57 ± 0.57	**(3.1)**[a]	0.056 ± 0.0004	(2.8)

Boldface indicates an increase greater than threefold.
[a] Increases in fold change were significant compared to TAM-1 without G335D or A371V.

Table 5
Drug susceptibilities of HIV-1 variants with G335D or A371V in the TAM-2 background.

Mutation	EC_{50} (μM) (fold increase)											
	AZT		d4T		ddI		3TC		ABC		TDF	
Wild Type	0.050 ± 0.002		2.55 ± 0.07		1.90 ± 0.17		0.45 ± 0.035		2.48 ± 0.21		0.020 ± 0.0023	
TAM-2	0.3960 ± 0.076	**(7.9)**	6.18 ± 0.11	(2.4)	6.71 ± 0.57	**(3.5)**	2.57 ± 0.089	**(5.7)**	2.97 ± 0.29	(1.2)	0.033 ± 0.0026	(1.7)
TAM-2/335D	2.6390 ± 0.396	**(52.7)**[a]	7.97 ± 0.47	**(3.1)**[a]	5.74 ± 0.63	**(3)**	2.37 ± 0.082	**(5.3)**	10.43 ± 0.41	**(4.2)**[a]	0.049 ± 0.0014	(2.4)[a]
TAM-2/371V	1.0600 ± 0.131	**(21.1)**[a]	8.29 ± 0.23	**(3.3)**[a]	6.00 ± 0.64	**(3.2)**	2.58 ± 0.072	**(5.8)**	3.43 ± 0.21	(1.4)	0.036 ± 0.0012	(1.8)
TAM-2/335D/371V	2.6340 ± 0.132	**(52.6)**[a]	13.71 ± 0.76	**(5.4)**[a]	13.76 ± 0.51	**(7.2)**[a]	2.45 ± 0.062	**(5.5)**	7.57 ± 0.21	**(3.1)**[a]	0.105 ± 0.0030	**(5.2)**[a]

Boldface indicates an increase greater than threefold.
[a] Increases in fold change were significant compared to TAM-2 without G335D or A371V.

subtype B (5.8%) and CRF01_AE (1.7%) infected individuals in the Stanford database. On the other hand, G335D, A371V and A400T were found in almost all the patients in our study. Although these three mutations are thought to be related to NRTI resistance in subtype B [7,11,16], they are common polymorphisms of wild-type CRF01_AE HIV-1 with prevalence of more than 90% in our previous study [20] and in the Stanford database. Therefore, we conclude that G335D, A371V and A400T detected in the present study were not selected after treatment but had existed before the introduction of treatment. Consequently, N348I was the only drug resistance mutation in the C-terminal half of RT observed in our cohort of treatment-experienced Vietnamese infected with CRF01_AE HIV-1.

Our results demonstrated that common CRF01_AE polymorphisms G335D and A371V play considerable role in drug resistance to NRTIs. Recent studies suggested that each of G335D or A371V is associated with drug resistance; G335D emerged after AZT exposure exhibits greater AZT resistance (8 to 53-fold over WT) when combined with TAM [11] and A371V selected in the background of D67N and K70R by high concentrations of AZT *in vitro* shows strong resistance to AZT in the presence of TAMs [7]. In agreement with those reports, our results showed that mutant containing G335D or A371V did not increase the resistance levels to NRTIs by themselves but they conferred higher resistance when combined with TAMs, especially to AZT (8.2–52.7 fold increase). Furthermore, we found that the dual mutation G335D/A371V had the greater impact than each single mutation on resistance in the presence of TAM. As G335D and A371V always appear together in treatment-naïve CRF01_AE, this finding is more critical for CRF01_AE HIV-1 infection than for subtype B infection. In addition, the fold change increased by G335D and A371V was greater with TAM-2 than that with TAM-1. Since TAM-2 is more frequent in CRF01_AE than in subtype B [28–30], this data is important for CRF01_AE HIV-1. Although the impact of G335D and A371V was the greatest in AZT resistance and seemed to be minor in other NRTIs' resistance, the fold-increase in TDF of G335D/A371V plus TAM-2 variant were above the clinical cut-off values [43], which can cause treatment failure. As TDF is often used in second line ART [2], this data is crucial for decisions on the next therapeutic strategies for CRF01_AE HIV-1-infected patients failing first line ART. Since our recombinant viruses were created with pBS-RT_{WT}, which was derived from subtype B RT but not from CRF01_AE RT, our results cannot be applied directly to CRF01_AE infection. CRF01_AE/B recombinants have been emerged and highly prevalent in Southeast Asian countries [32,44,45] and the breakpoint analysis showed some CRF01_AE/B recombinants consisted of subtype B N-terminal site and CRF01_AE C-terminal sites [45]. Therefore, our data suggests the potential influence of those CRF01_AE/B recombinants as well as CRF01_AE strain on the selection of second line therapy in Southeast Asia.

In summary, we reported the frequencies of drug resistance mutations in the connection subdomain and RNase H domain of RT in CRF01_AE HIV-1-infected Vietnamese who experienced ART. Then we demonstrated that the combination of G335D and A371V, a common pattern of polymorphisms in wild-type CRF01_AE, confer significant resistance to various NRTIs in the presence of TAMs. Our findings emphasize the important role of polymorphisms in C-terminal half of RT in CRF01_AE HIV-1 on drug resistance, especially in consideration of the second line therapy. Further investigation is needed on drug resistance mutations in widely prevailing non-subtype B HIV-1.

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References

[1] C.F. Gilks, S. Crowley, R. Ekpini, S. Gove, J. Perriens, Y. Souteyrand, D. Sutherland, M. Vitoria, T. Guerma, K. De Cock, The WHO public-health approach to antiretroviral treatment against HIV in resource-limited settings, Lancet 368 (2006) 505–510.
[2] Ministry of Health of Viet Nam, Decision by the Minister of Health on the Issuance of the Guidelines for HIV/AIDS Diagnosis and Treatment: Guidelines for HIV/AIDS Diagnosis and Treatment. Health Publishing House, 2005, No: 06/2005QD-BYT.

[3] UNAIDS/WHO, AIDS epidemic update ASIA regional summary 2007 Available from: http://data.unaids.org/pub/Report/2008/jc1527_epibriefs_asia_en.pdf (2008).
[4] UNAIDS/WHO, Epidemiological fact sheets on HIV/AIDS and sexually transmitted infections Available from: http://apps.who.int/GlobalAtlas/predefinedReports/EFS2008/index.asp (2008).
[5] Ministry of Health of Viet Nam, The third country report on following up the implementation to the declaration of commitment on HIV and AIDS Available from: http://data.unaids.org/pub/Report/2008/viet_nam_2008_country_progress_report_en.pdf (2008).
[6] D.E. Bennett, M. Myatt, S. Bertagnolio, D. Sutherland, C.F. Gilks, Recommendations for surveillance of transmitted HIV drug resistance in countries scaling up antiretroviral treatment, Antivir. Ther. 13 (2008) 25–36.
[7] J.H. Brehm, D. Koontz, J.D. Meteer, V. Pathak, N. Sluis-Cremer, J.W. Mellors, Selection of mutations in the connection and RNase H domains of human immunodeficiency virus type 1 reverse transcriptase that increase resistance to 3′-azido-3′-dideoxythymidine, J. Virol. 81 (2007) 7852–7859.
[8] K.A. Delviks-Frankenberry, G.N. Nikolenko, R. Barr, V.K. Pathak, Mutations in human immunodeficiency virus type 1 RNase H primer grip enhance 3′-azido-3′-deoxythymidine resistance, J. Virol. 81 (2007) 6837–6845.
[9] K.A. Delviks-Frankenberry, G.N. Nikolenko, P.L. Boyer, S.H. Huges, J. M. Coffin, A. Jere, V.K. Pathak, HIV-1 reverse transcriptase connection subdomain mutations reduce template RNA degradation and enhance AZT excision, Proc. Natl. Acad. Sci. USA 105 (2008) 10943–10948.
[10] A. Hachiya, E.N. Kodama, S.G. Sarafianos, M.M. Schuckmann, Y. Sakagami, M. Matsuoka, M. Takiguchi, H. Gatanaga, S. Oka, Amino acid mutation N348I in the connection subdomain of human immunodeficiency virus type 1 reverse transcriptase confers multiclass resistance to nucleoside and nonnucleoside reverse transcriptase inhibitors, J. Virol. 82 (2008) 3261–3270.
[11] G.N. Nikolenko, K.A. Delviks-Frankenberry, S. Palmer, F. Maldarelli, M.J. Fivash Jr., J.M. Coffin, V.K. Pathak, Mutations in the connection domain of HIV-1 reverse transcriptase increase 3′-azido-3′-deoxythymidine resistance, Proc. Natl. Acad. Sci. USA 104 (2007) 317–322.
[12] S.H. Yap, C.W. Sheen, J. Fahey, M. Zanin, D. Tyssen, V.D. Lima, B. Wynhoven, M. Kuiper, N. Sluis-Cremer, P.R. Harrigan, G. Tachedjian, N348I in the connection domain of HIV-1 reverse transcriptase confers zidovudine and nevirapine resistance, PLoS Med. 4 (2007) e335.
[13] M. Ehteshami, G.L. Beilhartz, B.J. Scarth, E.P. Tchesnokov, S. McCormick, B. Wynhoven, P.R. Harrigan, M. Götte, Connection domain mutations N348I and A360V in HIV-1 reverse transcriptase enhance resistance to 3′-azido-3′-deoxythymidine through both RNase H-dependent and -independent mechanisms, J. Biol. Chem. 283 (2008) 22222–22232.
[14] S. Zelina, C.W. Sheen, J. Radzio, J.W. Mellors, N. Sluis-Cremer, Mechanisms by which the G333D mutation in human immunodeficiency virus type 1 reverse transcriptase facilitates dual resistance to zidovudine and lamivudine, Antimicrob. Agents Chemother. 52 (2008) 157–163.
[15] J.H. Brehm, J.W. Mellors, N. Sluis-Cremer, Mechanism by which a glutamine to leucine substitution at residue 509 in the ribonuclease H domain of HIV-1 reverse transcriptase confers zidovudine resistance, Biochemistry 47 (2008) 14020–14027.
[16] K.A. Delviks-Frankenberry, G.N. Nikolenko, F. Maldarelli, S. Hase, Y. Takebe, V.K. Pathak, Subtype-specific differences in the human immunodeficiency virus type 1 reverse transcriptase connection subdomain of CRF01_AE are associated with higher levels of resistance to 3′-azido-3′-deoxythymidine, J. Virol. 83 (2009) 8502–8513.
[17] S.D. Kemp, C. Shi, S. Bloor, P.R. Harrigan, J.W. Mellors, B.A. Larder, A novel polymorphism at codon 333 of human immunodeficiency virus type 1 reverse transcriptase can facilitate dual resistance to zidovudine and L-2′,3′-dideoxy-3′-thiacytidine, J. Virol. 72 (1998) 5093–5098.
[18] M. Ntemgwa, M.A. Wainberg, M. Oliveira, D. Moisi, R. Lalonde, V. Micheli, B.G. Brenner, Variations in reverse transcriptase and RNase H domain mutations in human immunodeficiency virus type 1 clinical isolates are associated with divergent phenotypic resistance to zidovudine, Antimicrob. Agents Chemother. 51 (2007) 3861–3869.
[19] A.F. Santos, R.B. Lengruber, E.A. Soares, A. Jere, E. Sprinz, A.M. Martinez, J. Silveira, F.S. Sion, V.K. Pathak, M.A. Soares, Conservation patterns of HIV-1 RT connection and RNase H domains: identification of new mutations in NRTI-treated patients, PLoS ONE 3 (2008) e1781.
[20] A. Hachiya, K. Shimane, S.G. Sarafianose, E.N. Kodama, Y. Sakagami, F. Negishi, H. Koizumi, H. Gatanaga, M. Matsuoka, M. Takiguchi, S. Oka, Clinical relevance of substitutions in the connection subdomain and RNase H domain of HIV-1 reverse transcriptase from a cohort of antiretroviral treatment-naïve patients, Antiviral. Res. 82 (2009) 115–121.
[21] L. Buonaguro, M.L. Tornesello, F.M. Buonaguro, Human immunodeficiency virus type 1 subtype distribution in the worldwide epidemic: pathogenetic and therapeutic implications, J. Virol. 81 (2007) 10209–10219.
[22] R. Kantor, Impact of HIV-1 pol diversity on drug resistance and its clinical implications, Curr. Opin. Infect. Dis. 19 (2006) 594–606.
[23] R. Kantor, D.A. Katzenstein, B. Efron, A.P. Carvalho, B. Wynhoven, P. Cane, J. Clarke, S. Sirivichayakul, M.A. Soares, J. Snoeck, C. Pillay, H. Rudich, R. Rodrigues, A. Holguin, K. Ariyoshi, M.B. Bouzas, P. Cahn, W. Sugiura, V. Soriano, L.F. Brigido, Z. Grossman, L. Morris, A.M. Vandamme, A. Tanuri, P. Phanuphak, J.N. Weber, D. Pillay, P.R. Harrigan, R. Camacho, J.M. Schapiro, R.W. Shafer, Impact of HIV-1 subtype and antiretroviral therapy on protease and reverse transcriptase genotype: results of a global collaboration, PLoS Med. 2 (2005) e112.
[24] J. Snoeck, R. Kantor, R.W. Shafer, K. Van Laethem, K. Deforche, A.P. Carvalho, B. Wynhoven, M.A. Soares, P. Cane, J. Clarke, C. Pillay, S. Sirivichayakul, K. Ariyoshi, A. Holguin, H. Rudich, R. Rodrigues, M.B. Bouzas, F. Brun-Vézinet, C. Reid, P. Cahn, L.F. Brigido, Z. Grossman, V. Soriano, W. Sugiura, P. Phanuphak, L. Morris, J. Weber, D. Pillay, A. Tanuri, R.P. Harrigan, R. Camacho, J.M. Schapiro, D. Katzenstein, A.M. Vandamme, Discordances between interpretation algorithms for genotypic resistance to protease and reverse transcriptase inhibitors of human immunodeficiency virus are subtype dependent, Antimicrob. Agents Chemother. 50 (2006) 694–701.
[25] E. Caride, R. Brindeiro, K. Hertogs, B. Larder, P. Dehertogh, E. Machado, C.A. de Sá, W.A. Eyer-Silva, F.S. Sion, L.F. Passioni, J.A. Menezes, A.R. Calazans, A. Tanuri, Drug-resistant reverse transcriptase genotyping and phenotyping of B and Non-B subtypes (F and A) of human immunodeficiency virus type I found in Brazilian patients failing HAART, Virology 275 (2000) 107–115.
[26] B. Montes, L. Vergne, M. Peeters, J. Reynes, E. Delaporte, M. Segondy, Comparison of drug resistance mutations and their interpretation in patients infected with non-B HIV-1 variants and matched patients infected with HIV-1 subtype B, J. Acquir. Immune. Defic. Syndr. 35 (2004) 329–336.
[27] V. Novitsky, C.W. Wester, V. DeGruttola, H. Bussmann, S. Gaseitsiwe, A. Thomas, S. Moyo, R. Musonda, E. Van Widenfelt, R.G. Marlink, M. Essex, The reverse transcriptase 67N 70R 215Y genotype is the predominant TAM pathway associated with virologic failure among HIV Type 1C-infected adults treated with ZDV/ddI-Containing HAART in Southern Africa, AIDS Res. Hum. Retrovir. 23 (2007) 868–878.
[28] K. Ariyoshi, M. Matsuda, H. Miura, S. Tateishi, K. Yamada, W. Sugiura, Patterns of point mutations associated with antiretroviral drug treatment failure in CRF01_AE (subtype E) infection differ from subtype B infection, J. Acquir. Immune. Defic. Syndr. 33 (2003) 336–342.
[29] L.Y. Hsu, R. Subramaniam, L. Bacheler, N.I. Paton, Characterization of mutations in CRF01_AE virus isolates from antiretroviral treatment-naive and -experienced patients in Singapore, J. Acquir. Immune. Defic. Syndr. 38 (2005) 5–13.
[30] W.C. Yam, J.H. Chen, K.H. Wong, K. Chan, V.C. Cheng, H.Y. Lam, S.S. Lee, B.J. Zheng, K.Y. Yuen, Clinical utility of genotyping resistance test on determining the mutation patterns in HIV-1 CRF01_AE and subtype B patients receiving antiretroviral therapy in Hong Kong, J. Clin. Virol. 35 (2006) 454–457.
[31] C. Sukasem, V. Churdboonchart, W. Sukeepaisarncharoen, W. Piroj, T. Inwisai, M. Tiensuwan, W. Chantratita, Genotypic resistance profiles in antiretroviral-naive HIV-1 infections before and after initiation of first-line HAART: impact of polymorphism on resistance to therapy, Int. J. Antimicrob. Agents 31 (2008) 277–281.

[32] T.H.L. Nguyen, P. Recordon-Pinson, V.H. Pham, N.T. Uyen, T.T. Lien, H.T. Tien, I. Garrigue, M.H. Schrive, I. Pellegrin, M.E. Lafon, J.P. Aboulker, F. Barré-Sinoussi, H.J. Fleury, HIV type 1 isolates from 200 untreated individuals in Ho Chi Minh City (Vietnam): ANRS 1257 study. Large predominance of CRF01_AE and presence of major resistance mutations to antiretroviral drugs, AIDS Res. Hum. Retrovir. 19 (2003) 925–928.

[33] T.T.H. Tran, I. Maljkovic, S. Swartling, D.C. Phung, F. Chiodi, T. Leitner, HIV-1 CRF01_AE in intravenous drug users in Hanoi, Vietnam, AIDS Res. Hum. Retrovir. 20 (2004) 341–345.

[34] A. Ishizaki, H.C. Nguyen, P.V. Thuc, V.T. Nguyen, K. Saijoh, S. Kageyama, K. Ishigaki, J. Tanuma, S. Oka, H. Ichimura, Profile of HIV Type 1 infection and genotypic resistance mutations to antiretroviral drugs in treatment-naive HIV type 1-infected individuals in Hai Phong, Viet Nam, AIDS Res. Hum. Retrovir. 25 (2009) 175–182.

[35] A. Hachiya, S. Aizawa-Matsuoka, M. Tanaka, Y. Takahashi, S. Ida, H. Gatanaga, Y. Hirabayashi, A. Kojima, M. Tatsumi, S. Oka, Rapid and simple phenotypic assay for drug susceptibility of human immunodeficiency virus type 1 using CCR5-expressing HeLa/CD4$^+$ cell clone 1–10 (MAGIC-5), Antimicrob. Agents Chemother. 45 (2001) 495–501.

[36] V.A. Johnson, F. Brun-Vézinet, B. Clotet, H.F. Gunthard, D.R. Kuritzkes, D. Pillay, J.M. Schapiro, D.D. Richman, Update of the drug resistance mutations in HIV-1: December 2009, Top. HIV Med. 17 (2009) 138–145.

[37] Z. Hu, F. Giguel, H. Hatano, P. Reid, J. Lu, D.R. Kuritzkes, Fitness comparison of thymidine analog resistance pathways in human immunodeficiency virus type 1, J. Virol. 80 (2006) 7020–7027.

[38] R.W. Shafer, S.Y. Rhee, D. Pillay, V. Miller, P. Sandstrom, J.M. Schapiro, D.R. Kuritzkes, D. Bennett, HIV-1 protease and reverse transcriptase mutations for drug resistance surveillance, AIDS 21 (2007) 215–223.

[39] D.E. Bennett, R.J. Camacho, D. Otelea, D.R. Kuritzkes, H. Fleury, M. Kiuchi, W. Heneine, R. Kantor, M.R. Jordan, J.M. Schapiro, A.M. Vandamme, P. Sandstrom, C.A. Boucher, D. van de Vijver, S.Y. Rhee, T.F. Liu, D. Pillay, R.W. Shafer, Drug resistance mutations for surveillance of transmitted HIV-1 drug-resistance: 2009 update, PLoS ONE 4 (2009) e4724.

[40] S. Saeng-aroon, N. Tsuchiya, W. Auwanit, P.I. Ayuthaya, P. Pathipvanich, P. Sawanpanyalert, A. Rojanawiwat, M. Kannagi, K. Ariyoshi, W. Sugiura, Drug-resistant mutation patterns in CRF01_AE cases that failed d4T+3TC+nevirapine fixed-dosed, combination treatment: follow-up study from the Lampang cohort, Antivir. Res. 87 (2010) 22–29.

[41] S. Gupta, S. Fransen, E. Paxinos, W. Huang, R. Dua, E. Stawiski, C. Petropoulos, N. Parkin, Infrequent occurrence of mutations in the C-terminal region of reverse transcriptase modulates susceptibility to RT inhibitors, Antivir. Ther. 11 (2006) S143.

[42] E. Poveda, C. de Mendoza, T. Pattery, M. González, J. Villacian, V. Soriano, Phenotypic impact of resistance mutations on etravirine susceptibility in HIV patients with prior failure to nonnucleoside analogues, AIDS 22 (2008) 2395–2398.

[43] B. Winters, E. Van Craenenbroeck, K. Van der Borght, P. Lecocq, J. Villacian, L. Bacheler, Clinical cut-offs for HIV-1 phenotypic resistance estimates: update based on recent pivotal clinical trial data and a revised approach to viral mixtures, J. Virol. Methods 162 (2009) 101–108.

[44] B. Wang, K.A. Lau, L.Y. Ong, M. Shah, M.C. Steain, B. Foley, D.E. Dwyer, C.B. Chew, A. Kamarulzaman, K.P. Ng, N.K. Saksena, Complex patterns of the HIV-1 epidemic in Kuala Lumpur, Malaysia: evidence for expansion of circulating recombinant form CRF33_01B and detection of multiple other recombinants, Virology 367 (2007) 288–297.

[45] K.K. Tee, C.K. Pon, A. Kamarulzaman, K.P. Ng, Emergence of HIV-1 CRF01_AE/B unique recombinant forms in Kuala Lumpur, Malaysia, AIDS 19 (2005) 119–126.

INTERNAL MEDICINE

□ ORIGINAL ARTICLE □

Clinical Symptoms and Courses of Primary HIV-1 Infection in Recent Years in Japan

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Abstract

Background The natural course of HIV-1 infection includes 10 years of an asymptomatic period before the development of AIDS. However, in Japan, the disease progression process seems faster in recent years.

Methods The study subjects were 108 new patients with primary HIV-1 infection during the period from 1997 through 2007. We evaluated their clinical symptoms and laboratory data, and then analyzed disease progression in 82 eligible patients. Disease progression was defined as a fall in CD4 count below 350/μL and/or initiation of antiretroviral therapy.

Results Ninety percent of the patients were infected via homosexual intercourse. All patients had at least one clinical symptom (mean; 4.75±1.99) related to primary HIV-1 infection, with a mean duration of 23.2 days (±14.8) and 53.3% of them had to be hospitalized due to severe symptoms. The mean CD4 count and viral load at first visit were 390/μL (±220.1) and 4.81 log10/mL (±0.78), respectively. None developed AIDS during the study period. Estimates of risk of disease progression were 61.0% at 48 weeks and 82.2% at 144 weeks. In patients who required antiretroviral therapy, the median CD4 count was 215/μL (range, 52-858) at initiation of such therapy. Among the patients with a CD4 count of <350/μL at first visit, 53% never showed recovery of CD4 count (>350/μL) without antiretroviral therapy.

Conclusion Despite possible bias in patient population, disease progression seemed faster in symptomatic Japanese patients with recently acquired primary HIV-1 infection than the previously defined natural course of the disease.

Key words: HIV-1, primary infection, disease progression

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Introduction

The natural course of HIV-1 infection has been well described in large cohorts from the United States and Europe before the introduction of highly active antiretroviral therapy (HAART); primary HIV-1 infection (PHI) is followed by a clinical latency, usually lasting around 10 years, which precedes the eventual collapse of the immune system (1, 2). However, there is a common feeling among clinicians at present that the natural disease progression of recently infected patients is faster than in previous years (3, 4). Disease progression depends on various factors such as HLA type (5), concomitant infections (6, 7), and available medical resources (8). In addition to these factors, events occurring during PHI could also determine the natural course of the disease. Initial studies suggested that patients with more symptoms related to primary PHI and longer duration of illness exhibit faster rates of progression to AIDS (9-13). Plasma viral load at a set point is also an independent predictor of disease progression (14, 15). However, to determine the viral set point is sometimes difficult. Therefore, for clinicians, the severity of clinical symptoms is the only predictor of subsequent disease progression. The latency be-

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tween the development of PHI and commencement of HAART is also important in the present HAART era.

The main aim of this study was to evaluate the natural disease progression of recently infected Japanese patients. To determine whether or not the disease progression of recently infected patients is accelerated, their CD4 decline was compared with that of hemophiliacs infected before 1985 as the first HIV-1 infection in Japanese.

Furthermore, we also evaluated the correlation between initial CD4 count, viral load, and clinical events and subsequent changes in CD4 and/or time to start HAART in symptomatic Japanese patients with PHI.

Patients and Methods

Study site and patients with PHI

This study was conducted at the AIDS Clinical Center (ACC), National Center for Global Health and Medicine (NCGM: formerly International Medical Center of Japan). The NCGM (925 beds) is a tertiary general hospital located in central Tokyo and the ACC is the main referral clinic for treatment of HIV infected patients in Japan. As part of the follow-up service, HIV-1 infected patients usually visit the ACC on a monthly basis and CD4 count and viral load are measured at each visit. In the present retrospective study, we reviewed the medical records of 108 patients with PHI who were newly diagnosed with PHI between 1997 through 2007 at the ACC. We had conducted a clinical trial of structured treatment interruptions in patients with PHI from November 2000 through December 2002 and 26 patients were enrolled in that trial (16, 17). In terms of the data of these 26 patients, only the initial clinical and laboratory data were included in the present analysis, while all other data, such as time to events, were excluded from this study. To compare the natural CD4 decline of previously and recently infected patients, CD4 counts of 42 Japanese hemophiliacs recorded in the database in 1988 were analyzed as a previous control. Japanese hemophiliacs were infected with HIV-1 through contaminated blood products before 1985 (the estimated mean year of infection was 1983). Therefore, CD4 counts at the end of 1988 were the data at least 3 years after infection. In this comparison, the number of eligible recently infected patients was 59 patients; untreated and CD4 count at 3 years after infection was available.

Definition of PHI

PHI was diagnosed based on the presence of the following three criteria: 1) negative or incomplete western blot finding at the first visit with subsequent change to positive, 2) negative or weakly reactive enzyme-linked immunosorbent assay (ELISA) result for plasma HIV-1 RNA, and 3) confirmed HIV-1 infection on the first visit with documentation of negative ELISA result within 6 months. Symptomatic PHI was defined as PHI accompanied by at least one symptom related to acute retroviral syndrome, such as fever, lymphadenopathy, or skin rash.

Definition of disease progression

Disease progression was defined as fall in CD4 count below 350/μL and/or initiation of antiretroviral therapy. Specifically, patients with an AIDS-defined illness [listed under Centers for Disease Control and Prevention (CDC) category C], patients with AIDS requiring initiation of HAART, and those with severe symptomatic PHI on HAART were defined to have disease progression. The selection of a cutoff value of 350/μL for CD4 count was based on the fact that treatment is generally indicated during the chronic phase of infection when CD4 count falls below 350/μL (18). Patients were considered to be in immunologic progression at the first visit when the initial CD4 count was <350/μL and never subsequently reached 350/μL. For patients who showed a spontaneous increase in subsequent CD4 counts to ≥350/μL (such recovery occurred within 3 months from the first visit in all such patients), disease progression was set to have started at the time when such change in CD4 count occurred.

Statistical analysis

Continuous variables are presented as mean value ± SD. Categorical variables were presented as absolute numbers and proportions. Time to events was analyzed by the Kaplan-Meier survival curves, and compared using log-rank test. For patients who did not experience the events described above, data were censored at their last visit. To evaluate the differences between patients groups, the Student t test and χ^2 test were used when appropriate. The relationships between variables were analyzed by the Spearman rank-over correlation test. Statistical significance was defined as $p<0.05$. Data were analyzed using SPSS for Windows (version 15, SPSS, Inc., Chicago, IL).

Results

Table 1 lists the demographics of the enrolled patients with PHI. All patients had at least one documented symptom consistent with PHI (median 5; range 1-11). Fever, cervical lymphadenopathy, pharyngitis, and rash were found in more than 50% of patients (Table 2). The mean duration of symptoms was 23.2 days (SD ±14.8). Fifty-eight (53.7%) patients had to be hospitalized due to severe clinical symptoms. The initial viral loads in hospitalized patients were significantly higher than those of non-hospitalized patients. A longer duration of symptoms was associated with higher initial viral load (R=0.31, p=0.002) (Fig. 1A), and lower CD 4 count (R=-0.22, p=0.03) (Fig. 1B). Consequently, a higher viral load slightly was correlated with a lower CD4 count at the first visit (R=-0.22, p=0.033) (Fig. 1C).

Disease progression was analyzed in 82 patients. None of the patients had AIDS-defining events. Estimates of the risk of disease progression were 50.6% at 24 weeks, 61.0% at 48 weeks, 67.0% at 96 weeks, and 82.2% at 144 weeks

Table 1. Baseline Characteristics of 108 Patients with Primary HIV-1 Infection in this Study

Characteristics	Total number or mean (±SD) or %	Hospitalized patients (n = 58)	Non-hospitalized patients (n = 50)	p
Age (year)	31.8±8.48	32±9.07	31±7.82	NS
Sex				
Male	102	56	46	NS
Female	6	2	4	NS
Predisposing factor				
MSM	97	53	44	NS
Heterosexual	8	3	5	NS
IDU	1	0	1	NS
Unknown	2	2	0	NS
PMH of STD	75 (69.7)	44 (40.4)	31 (29.3)	NS
Syphilis	49 (45.5)	27 (25.3)	21 (20.2)	NS
Acute hepatitis A	11 (10.1)	6 (6.1)	5 (4.0)	NS
Acute hepatitis B	36 (33.3)	22 (20.2)	14 (13.1)	NS
Amebiasis	10 (9.1)	9 (8.0)	1 (1.1)	0.035
Others	7 (6.1)	2 (2.0)	5 (4.1)	NS
No. of symptoms	4.75±1.99	4.98±1.94	4.48±2.04	NS
Duration of symptoms (days)	23.2±14.8	27.8±13.1	18.0±15.1	0.001
Laboratory findings				
CD4 count/μL	390.0±220.1	356.1±204.1	443.7±236.0	0.06
HIV RNA $\log_{10}$/mL	4.81±0.78	5.03±0.68	4.48±0.81	0.001
STI trial*	26	12	14	NS

*Patients enrolled in a clinical trial of structured treatment interruptions in recently HIV-1-infected patients. Abbreviations; MSM: men who have sex with men, PMH of STD: past medical history of sexual transmitted diseases, STI: structured treatment interpretations, IDU: intravenous drug user, Others: genital herpes infection, chlamydial urethral infection condyloma acuminata, NS: not significant

Data are presented as mean ± SD or percentage (%) unless otherwise indicated

Table 2. Symptoms and Physical Findings Observed in the Patients with >10% Frequencies (n=108)

Symptoms and physical findings	frequency (%)
Fever	91
Lymphadenopathy	63
Pharyngitis	53
Rash	50
Diarrhea	37
Fatigue	32
Headache	26
Myalgia	20
Weight loss	19
Nausea	16
Appetite loss	14
Neurological sign	13
Hepatomegaly	13
Thrush	12

(Fig. 2). Eighteen of 34 (53.3%) patients with an initial CD 4 cell count below 350 cells/μL had immunologic progression at the first visit. Their CD4 counts never increased above 350/μL until initiation of HAART. Forty-eight (58.5%) required initiation of HAART in this study. The reasons for the initiation of HAART were severe clinical symptoms related to PHI in 16 patients and immunologic progression in 32 patients. The median CD4 count of those patients at initiation of HAART was 215/μL (range, 52-858).

We analyzed the clinical course in 66 patients (excluding 26 patients who enrolled in a clinical trial of structured treatment interruptions in PHI and 16 patients who received HAART for PHI) to determine the factors associated with disease progression. Half of these patients (33 patients) required hospitalization. As shown in Fig. 3A, the mean time to disease progression of the hospitalized patients [57.4 weeks, 95% confidence interval (95%CI); 34.9-79.8 weeks] was shorter than that of the non-hospitalized (33 patients, 94.4 weeks, 95%CI; 71-117 weeks, p=0.002). Among the 32 patients with CD4 count >350/μL at first visit, 24% had documented disease progression within 1 year, whereas among 34 patients with CD4 count <350/μL at first visit, 76.4% showed disease progression (Fig. 3B). The mean times to disease progression for the two groups were 111.9 weeks (95%CI; 92.8-131) and 39.5 weeks (95%CI; 18.6-60.5), respectively (p<0.001). Disease progression in 39 patients with high viral load (≥5.0 $\log_{10}$/mL) was not significantly different (p=0.41) from that in 27 patients with low viral load (<5.0 $\log_{10}$/mL) (Fig. 3C). The number of symptoms was not significantly different in each group (Fig. 3D). The mean time to disease progression was 69.8 weeks (95% CI; 47.2-92.5) in patients with a high viral load and 80.4 weeks (95%CI; 54.9-105.8) in those with a low viral load.

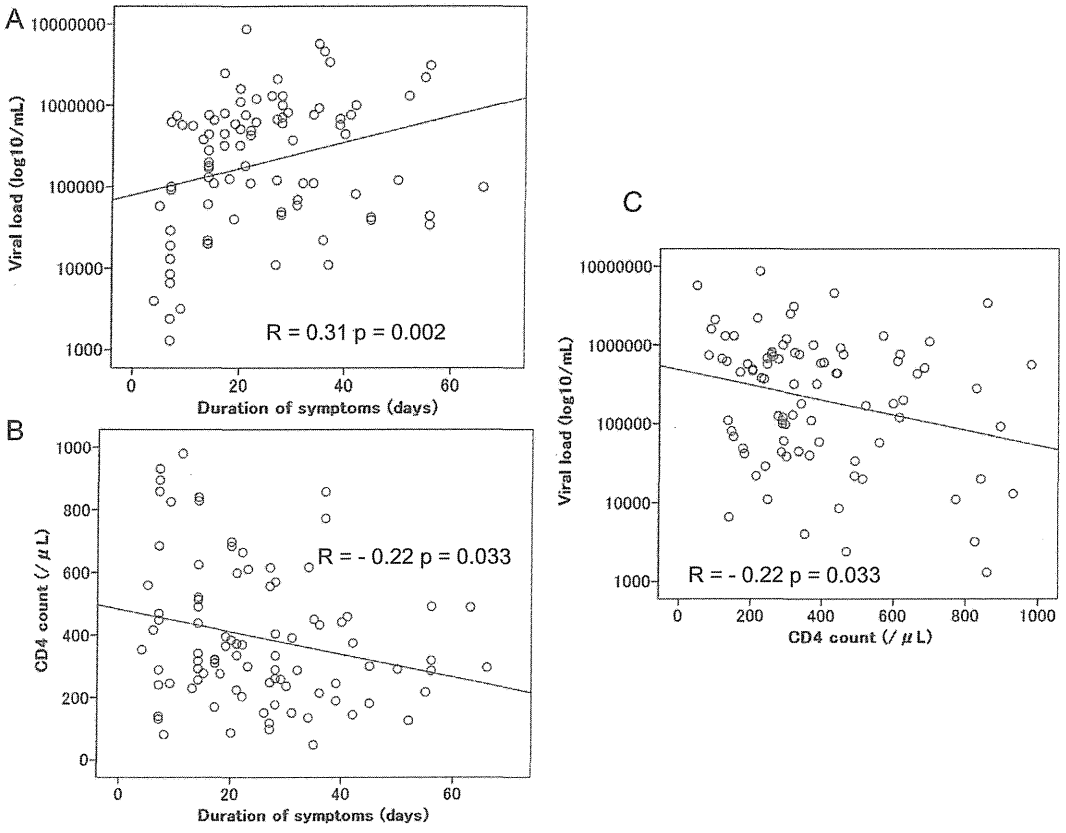

Figure 1. **Correlations among plasma viral load, CD4 count, and clinical symptoms. A; Plasma viral load correlated with duration of symptoms (R=0.31, p=0.002). B; CD4 count correlated inversely with duration of symptoms (R=-0.22, p=0.033). C; plasma viral load correlated inversely with CD4 count (R=-0.22, p=0.033).**

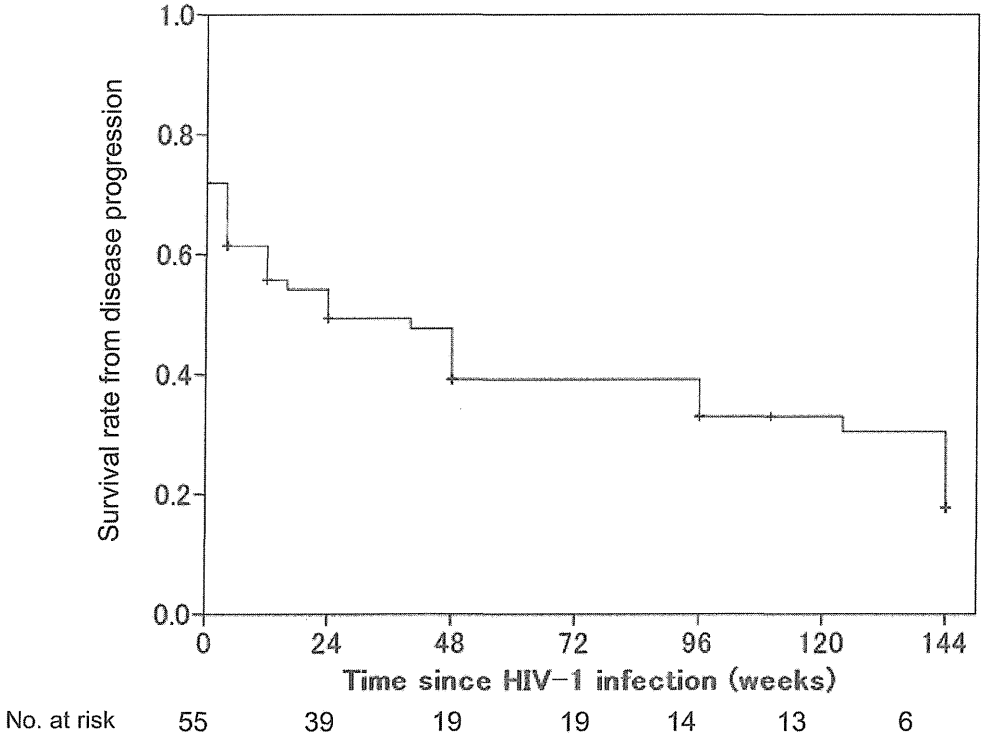

Figure 2. **Progression-free survival in 82 patients. Progression was defined as CD4 count <350/μL or initiation of HAART. No. at risk: the number of CD4 count >350/μL or HAART naïve patients**

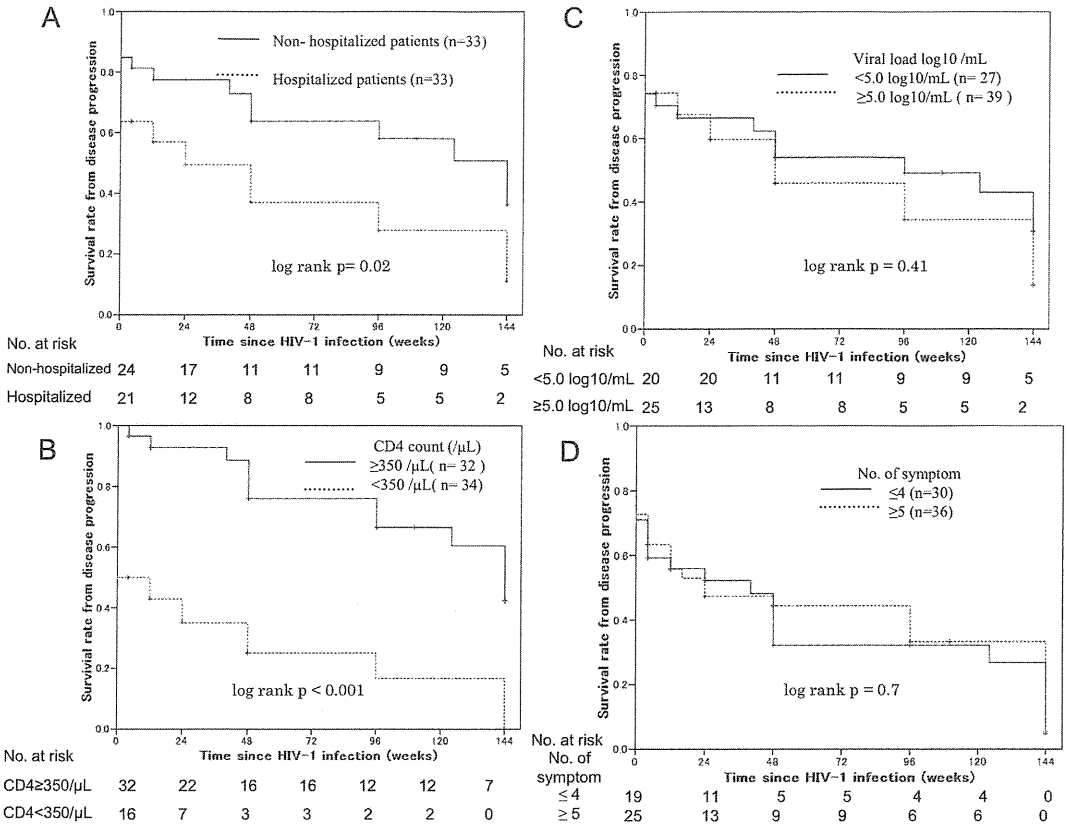

Figure 3. Progression-free survival among 66 patients according to rate of hospitalization, baseline CD4 count, and viral load. No. at risk: the number of CD4 count >350/μL or HAART naïve patients. A; Solid line: patients who required hospitalization due to PHI, dashed line: patients who did not require hospitalization (p=0.02, by log-rank test). B; Solid line: patients with CD4 count >350/μL at first visit, dashed line: patients with CD4 count <350/μL (p<0.001). C; Solid line: patients with viral load <5.0 log_{10}/mL, dashed line: patients with viral load ≥5.0 log_{10}/mL (p=0.41). Disease progression was defined as CD4 count <350/μL or initiation of HAART. D; Solid line: patients with the number of PHI symptoms ≤4, dashed line: patients with the number of PHI symptoms ≥5 (p=0.7, by log-rank test).

Comparison of percentage of recently infected patients with CD4 counts >350/μL at 3 years after infection and that of hemophiliacs as the first HIV-1 infected population in Japanese is shown in Fig. 4. The percentage (13.5%) of recently infected patients was significantly lower than that (47.6%) of Japanese hemophiliacs (p<0.001), clearly indicating the rapid decline of CD4 count in recently infected patients.

Discussion

In this study, we demonstrated rapid disease progression of symptomatic PHI Japanese patients in this decade. However, when we divided our study subjects into two groups according to the first half (1997-2002) and the latter half (2003-2007), disease progression of each group was not different (data not shown). In contrast, disease progression surrogated with natural CD4 decline of recently infected patients was significantly accelerated compared with Japanese hemophiliacs infected with HIV-1 before 1985. However, there are two quite different backgrounds; one is the route of infection and the other is the year of infection. Almost all hemophiliac patients are also co-infected with hepatitis C but do not have other sexually transmitted diseases (STDs). In contrast, most patients in the present study were infected via homosexual intercourse with many other STDs that may facilitate acceleration of the disease progression (7). In the present study, 69.7% patients had a past medical history of STDs, and the mean number of STDs was 1.08/patient (0: 31.3%, 1: 37.4%, 2: 23.2%, 3: 8.1%). In this regard, most published data on disease progression were obtained from men who have sex with men (MSM) cohorts (1, 2). Therefore, it is unlikely that the recent rapid disease progression is due to Japanese MSM. Whether or not the rapid disease progression in the recently HIV-1-infected Japanese can be generalized is to be elucidated in future studies.

Some HLA types are protective against disease progression such as HLA-B57 (19) and HLA-B51 (20) because HLA-restricted cytotoxic T lymphocytes (CTLs) play an important role on viral control. On the other hand, virus can easily escape from CTLs (17, 21). In some prevalent HLA types, escape virus can transmit and accumulate in the population (21). In this situation, some HLA types are no more

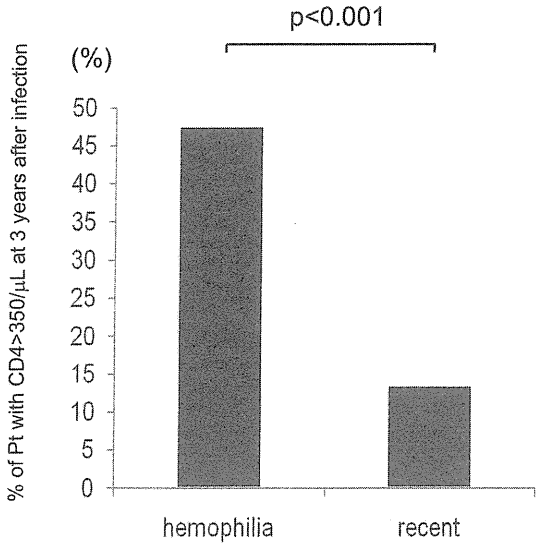

Figure 4. Comparison of percentage of previously and recently infected patients with CD4 counts >350/μL at 3 years after infection. In this analysis, Japanese hemophiliacs (designated "hemophilia" in the figure) were regarded as a previously infected patient, because they were infected with HIV-1 before 1985. The number of hemophiliacs was 42 patients. The eligible number of recently infected patients (designated "recent" in the figure) was 59 patients; infected with HIV-1 after 1997, untreated, and CD4 count at 3 years after infection.

protective. The HLA distribution is different in Americans compared to Japanese. Another possible hypothesis for the different disease progression is that Japanese hemophiliacs were exposed to HIV-1 through contaminated blood products imported from US as the first Japanese population infected with the virus around 1983. However, in recent years, most HIV-1 infection in Japanese is transmitted from Japanese patients. It can be postulated that current HIV-1 in Japan has adapted to the Japanese population, indicating acquisition and accumulation of escape virus from immune pressure of the otherwise protective HLA in Japanese population (21). From a negative point of view, the situation is similar to the epidemic of drug-resistance virus in treatment of naïve patients (22). The clinical relevance of the prevalence of immune escape virus in Japanese is a potentially serious matter in terms of the natural course of HIV-1 infection.

In the present study, all patients have had at least one symptom associated with PHI. During the follow-up period, no patient developed AIDS, whereas around 70% of the patients experienced immunologic progression as defined by a CD4 count <350/μL. It is noteworthy that the majority of these patients exhibited immunologic progression within 3 years and, surprisingly, >60% of them were documented within the first year. HAART was initiated in nearly 60% of patients during this period, including initiation for PHI-related severe symptoms in 20% of these patients. Previous studies on PHI have suggested that the number, duration, and/or severity of symptoms can predict faster disease progression to AIDS (23, 24). Our findings are compatible with these previous studies. Considered together, these results suggest that the duration of illness rather than the number of symptoms is more likely to be a major determinant of immunological progression. The estimated risks of disease progression were more than 50% by week 24 and 80% by week 144. Comparison with those observed elsewhere during the natural course of HIV-1 infection (24), these disease progression rates are surprisingly high. Among the patients with CD4 counts >350/μL at first visit, a quarter of them showed disease progression within 1 year. In contrast, in patients with CD4 count <350/μL, three quarters of them showed disease progression within the same period. Goujard et al (25) suggested possible recovery of CD4 count after the primary infection phase even in patients with very low count because it fluctuates during that period. In contrast, our results suggest that patients with a CD4 count of <350/μL during primary infection should be monitored carefully because spontaneous recovery of CD4 cell count during primary infection was rare. This cautionary remark could also apply to patients with a CD4 count of >350/μL because they exhibited nearly 60% risk of disease progression within 3 years. These observations may allow more targeted clinical monitoring and timely initiation of HAART. The impact of a short-term HAART during symptomatic primary infection on the subsequent disease progression needs to be elucidated in future study.

Although we included all recent seroconverters during the study period, it could be argued that this study carries some institution bias (i.e., a high proportion of cases with severe disease). However, the present finding of a surprisingly rapid disease progression in our patient population is new. Whether or not the natural course of disease progression has recently become accelerated in other countries or other cohorts is a matter of great interest.

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

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References

1. Collaborative Group on AIDS Incubation and HIV Survival. Time from HIV-1 seroconversion to AIDS and death before widespread use of highly-active antiretroviral therapy: a collaborative reanalysis. Lancet **355**: 1131-1137, 2000.
2. Lyles RH, Munoz A, Yamashita TE, et al. Natural history of human immunodeficiency virus type 1 viremia after seroconversion and proximal to AIDS in a large cohort of homosexual men. Multicenter AIDS cohort study. J Infect Dis **181**: 872-880, 2000.
3. Crum-Cianflone N, Eberly L, Zhang Y, et al. Is HIV becoming more virulent? Initial CD4 counts among HIV seroconverters during the course of the HIV epidemic: 1985-2007. Clin Infect Dis **48**: 1285-1292, 2009.

4. Muller V, Maggiolo F, Suter F, et al. Increasing clinical virulence in two decades of the Italian HIV epidemic. PLoS Pathog **5**: e1000454, 2009.
5. Carrington M, O'Brien SJ. The influence of HLA genotype on AIDS. Annu Rev Med **54**: 535-551, 2003.
6. McShane H. Co-infection with HIV and TB: double trouble. Int J STD AIDS **16**: 95-100, 2005.
7. Palacios R, Jimenez-Onate F, Aguilar M, et al. Impact syphilis infection on HIV viral load and CD4 cell counts in HIV-infected patients. J Acquir Immun Defic Syndr **44**: 356-359, 2007.
8. Duncombe C, Kerr SJ, Ruxrungtham K, et al. HIV disease progression in a patient cohort treated via a clinical research network in a resource limited setting. AIDS **28**: 169-178, 2005.
9. Pedersen C, Katzenstein T, Nielsen C, et al. Prognostic value of serum HIV-RNA levels at virologic steady state after seroconversion: relation to CD4 cell count and clinical course of primary infection. J Acquir Immune Defic Syndr Hum Retrovirol **16**: 93-99, 1997.
10. Vanhems P, Hirschel B, Phillips AN, et al. Incubation time of acute human immunodeficiency virus (HIV) infection and duration of acute HIV infection are independent prognostic factors of progression to AIDS. J Infect Dis **182**: 334-337, 2000.
11. Vanhems P, Lanbert J, Cooper DA, et al. Severity and prognosis of acute human immunodeficiency virus type 1 illness: a dose-response relationship. Clin Infect Dis **26**: 323-329, 1998.
12. Sterling T, Vlahov D, Astemborski J, et al. Initial plasma HIV-1 RNA levels and progression to AIDS in women and men. N Engl J Med **344**: 720-725, 2001.
13. Henrard DR, Daar E, Farzadegan H, et al. Virologic and immunologic characterization of symptomatic and asymptomatic primary HIV-1 infection. J Acquir Immune Defic Syndr Hum Retrovirol **9**: 305-310, 1995.
14. Schacker TW, Hughes JP, Shea T, et al. Biological and virologic characteristics of primary HIV infection. Ann Intern Med **128**: 613-620, 1998.
15. Lefrere JJ, Roudot-Thoraval F, Mariotti M, et al. The risk of disease progression is determined during the first year of human immunodeficiency virus type 1 infection. J Infect Dis **177**: 1541-1548, 1998.
16. Fujiwara M, Tanuma J, Koizumi H, et al. Different abilities of escape mutant-specific cytotoxic T cells to suppress replication of escape mutant and wild-type human immunodeficiency virus type 1 in new hosts. J Virol **82**: 138-147, 2008.
17. Tanuma J, Fujiwara M, Teruya K, et al. HLA-A*2402-restricted HIV-1 specific T lymphocytes and escape mutation after ART with structured treatment interruptions. Microbes Infect **10**: 689-698, 2008.
18. Thompson MA, Aberg JA, Cahn P, et al. Antiretroviral treatment of adult HIV infection; 2010 recommendations of the international AIDS Society-USA Panel. JAMA **304**: 321-333, 2010.
19. Goulder PJ, Bunce M, Krausa P, et al. Novel, cross-restricted, conserved, and immunodominant cytotoxic T lymphocyte epitopes in slow progressors in HIV type 1 infection. AIDS Res Hum Retroviruses **10**: 1691-1698, 1996.
20. Kawashima Y, Kuse N, Gatanaga H, et al. Long-term control of HIV-1 in hemophiliacs carrying slow-progressing allele HLA-B* 5101. J Virol **84**: 7151-7160, 2010.
21. Kawashima Y, Pfafferott K, Frater J, et al. Adaptation of HIV-1 to human leukocyte antigen class I. Nature **458**: 641-645, 2009.
22. Little SJ, Holte S, Routy JP, et al. Antiretroviral-drug resistance among patients recently infected with HIV. N Engl J Med **347**: 385-394, 2002.
23. Vanhems P, Lambert J, Cooper DA, et al. Severity and prognosis of acute immunodeficiency virus type 1 illness: a dose-response relationship. Clin Infect Dis **26**: 323-329, 1998.
24. Lavreys L, Baeten JM, Chohan V, et al. Higher set point plasma viral load and more-severe acute HIV type 1 (HIV-1) illness predict mortality among high-risk HIV-1-infected African women. Clin Infect Dis **42**: 1333-1339, 2006.
25. Goujard C, Bonarek M, Meyer L, et al. CD4 cell count and HIV DNA level are independent predictors of disease progression after primary HIV type 1 infection in untreated patients. Clin Infect Dis **42**: 709-715, 2006.