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Long-Term Control of HIV-1 in Hemophiliacs Carrying Slow-Progressing Allele HLA-B*5101▽†

Yuka Kawashima,[1]‡ Nozomi Kuse,[1]‡ Hiroyuki Gatanaga,[2,3]‡ Takuya Naruto,[1] Mamoru Fujiwara,[1] Sachi Dohki,[1] Tomohiro Akahoshi,[1] Katsumi Maenaka,[4] Philip Goulder,[5] Shinichi Oka,[2,3] and Masafumi Takiguchi[1]*

Divisions of Viral Immunology[1] and Infectious Disease,[2] Center for AIDS Research, Kumamoto University, 2-2-1 Honjo, Kumamoto 860-0811, AIDS Clinical Center, National Center for Global Health and Medicine, 1-21-1 Toyama, Shinjuku, Tokyo 162-8655,[3] and Medical Institute of Bioregulation, Kyushu University, 3-1-1 Maidashi, Fukuoka,[4] Japan, and Department of Paediatrics, Peter Medawar Building for Pathogen Research, Oxford University, Oxford OX1 3SY, United Kingdom[5]

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HLA-B*51 alleles are reported to be associated with slow disease progression to AIDS, but the mechanism underlying this association is still unclear. In the present study, we analyzed the effect of HLA-B*5101 on clinical outcome for Japanese hemophiliacs who had been infected with HIV-1 before 1985 and had been recruited in 1998 for this study. HLA-B*5101$^+$ hemophiliacs exhibited significantly slow progression. The analysis of HLA-B*5101-restricted HIV-1-specific cytotoxic T-lymphocyte (CTL) responses to 4 HLA-B*-restricted epitopes in 10 antiretroviral-therapy (ART)-free HLA-B*5101$^+$ hemophiliacs showed that the frequency of Pol283-8-specific CD8$^+$ T cells was inversely correlated with the viral load, whereas the frequencies of CD8$^+$ T cells specific for 3 other epitopes were positively correlated with the viral load. The HLA-B*5101$^+$ hemophiliacs whose HIV-1 replication had been controlled for approximately 25 years had HIV-1 possessing the wild-type Pol283-8 sequence or the Pol283-8V mutant, which does not critically affect T-cell recognition, whereas other HLA-B*5101$^+$ hemophiliacs had HIV-1 with escape mutations in this epitope. The results suggest that the control of HIV-1 over approximately 25 years in HLA-B*5101-positive hemophiliacs is associated with a Pol283-8-specific CD8$^+$ T-cell response and that lack of control of HIV-1 is associated with the appearance of Pol283-8-specific escape mutants.

Human immunodeficiency virus type 1 (HIV-1)-specific CD8$^+$ T cells play a critical role in the control of HIV-1 infections (26, 5), but HIV-1 escape occurs during acute and chronic phases of an HIV-1 infection (6, 14). There are several mechanisms affording HIV-1 escape from the host immune system. They include the appearance of mutants that escape from HIV-1-specific cytotoxic T lymphocytes (CTLs) (6, 14) and neutralizing antibodies (27, 47, 48), impaired recognition of HIV-1-infected cells by HIV-1-specific CTLs due to Nef-mediated downregulation of HLA class I molecules (8, 42), and impaired function of HIV-1-specific T cells (3).

It is well known that long-term nonprogressors (LTNPs), who remain disease free and have very low or undetectable viral loads (VLs) in the absence of antiretroviral therapy (ART), exist as a very small population of HIV-1-infected individuals (7, 21, 38). A small minority of these LTNPs were infected by HIV-1 containing deletions in viral accessory molecules (10, 17, 24). HLA alleles such as HLA-B*57/5801, HLA-B*27, and HLA-B*51 are associated with slow progression to AIDS (19, 22, 37). Indeed, it is reported that many LTNPs carry these HLA alleles (31, 36). These findings imply that HIV-1-specific CTLs restricted by these alleles may play an important role in the control of HIV-1 replication in LTNPs. The mechanism of control of HIV-1 replication has been analyzed in LTNPs and slow progressors carrying HLA-B*57/5801, HLA-B*27, or HLA-B*13, and has been related to the Gag-specific CD8$^+$ T-cell epitopes presented by these alleles (9, 11, 14, 16, 34). On the other hand, the mechanism underlying the association between HLA-B*5101 and slow progression remains unclear. To date, no study of the mechanism of control of HIV-1 in HLA-B*5101$^+$ LTNPs has been reported.

Since the data indicate that HIV-1 replication can be controlled for more than 20 years in LTNP hemophiliacs, analysis of HIV-1-specific immune responses and HIV-1 in these patients is useful for investigating the immunological control of HIV-1. In Japan, HLA-B*57/58 and HLA-B*27 are very rare alleles (18). Therefore, it was speculated that only HLA-B*51 would play an important role in the control of HIV-1 replication in HIV-1-infected Japanese donors.

We showed previously that 2 Pol peptides and 1 Gag peptide were HLA-B*5101-restricted immunodominant CTL epitopes (45). Two Pol-specific CTLs are known to have strong abilities to suppress HIV-1 replication *in vitro* (43). Our recent study using 9 cohorts showed that of these T cells, Pol283-specific CTLs select mutations at position 8 (position 135 of reverse transcriptase [RT]) in the epitope (20). A Thr mutation at position 8 (8T) was found predominantly in HIV-1-infected HLA-B*5101$^+$ donors, whereas the 8R, 8L, and 8V mutations were also found in these donors. The 8T, 8L, and 8R mutants had fitness similar to

* Corresponding author. Mailing address: Division of Viral Immunology, Center for AIDS Research, Kumamoto University, 2-2-1 Honjo, Kumamoto 860-0811, Japan. Phone: 81-96-373-6529. Fax: 81-96-373-6532. E-mail: masafumi@kumamoto-u.ac.jp.

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‡ Y.K., N.K., and H.G. contributed equally to this work.

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that of the wild-type virus, whereas the 8V mutation had a higher fitness cost than the others.

In the present study, we analyzed the effect of HLA-B*5101 on clinical outcome in Japanese hemophiliacs infected with HIV-1. In addition, we investigated the role of HLA-B*5101-restricted HIV-1-specific CTLs *in vivo* in HLA-B*5101$^+$ LTNP and slow-progressing Japanese hemophiliacs who had not been treated with antiretroviral therapy for approximately 25 years. Our results revealed a role for Pol283-8-specific HLA-B*5101-restricted HIV-1-specific CTLs in the long-lasting (approximately 25 years) control of HIV-1 replication.

MATERIALS AND METHODS

Patients. One hundred eight Japanese hemophiliacs who had been infected with HIV-1 before 1985, mostly around 1983, were recruited for the present study, which was approved by the ethics committees of Kumamoto University and the National Center for Global Health and Medicine. Written informed consent was obtained from all subjects according to the Declaration of Helsinki. Patient HLA type was determined by standard sequence-based genotyping. For sequence analysis, blood specimens were collected in EDTA. Plasma and peripheral blood mononuclear cells (PBMCs) were separated from heparinized whole blood.

Cells. C1R and 721.221 cells expressing HLA-B*5101 (C1R-B*5101 and 721.221-B5101, respectively) were generated previously (15, 33, 44). All cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and 0.15 mg/ml hygromycin B.

HIV-1 clones. An infectious proviral clone of HIV-1, pNL-432, and its mutant, pNL-M20A (containing a substitution of Ala for Met at residue 20 of Nef), were reported previously (1). Pol283-8 and Pol743-9 mutant (Pol283-8L, -8T, -8V, and -8R; Pol743-11, -5I, and -4151) viruses were generated based on pNL-432 by using the GeneTailor site-directed mutagenesis system (Invitrogen).

HLA class I tetramers. HLA class I-peptide tetrameric complexes (tetramers) were synthesized as described previously (2). Four HIV-1 specific epitopes (Pol283-8, Pol743-9, Gag327-8, and Rev71-11) (45) were used for the refolding of HLA-B*5101 molecules. Phycoerythrin (PE)-labeled streptavidin (Molecular Probes) was used for the generation of the tetramers.

Flow cytometric analysis using tetramers. PBMCs were incubated with the tetramers at 37°C for 30 min. The cells were subsequently washed twice with RPMI–10% newborn calf serum (NCS) and were then stained with an anti-CD8 monoclonal antibody (MAb). Next, they were incubated at 4°C for 30 min and were then washed twice with RPMI–10% NCS. The cells were finally resuspended in phosphate-buffered saline (PBS) containing 2% paraformaldehyde, and then the percentage of tetramer-positive cells among the CD8$^+$ population was determined by using a FACSCalibur flow cytometer (BD Bioscience, San Jose, CA).

Generation of CTL clones. Pol283-8-specific CTL clones and Pol743-9-specific CTL clones were generated from HIV-1-specific bulk-cultured T cells by limiting dilution in U-bottom 96-well microtiter plates (Nunc, Roskilde, Denmark) containing 200 μl of cloning mixture (about 1×10^6 irradiated allogeneic PBMCs from healthy donors and 1×10^5 irradiated C1R-B*5101 cells prepulsed with the corresponding peptide at 1 μM in RPMI 1640 supplemented with 10% human plasma and 200 U/ml human recombinant interleukin-2 [rIL-2]) (43).

CTL assay for target cells infected with HIV-1. The cytotoxicity of CTL clones for 721.221-B5101 cells infected with HIV-1 (>30% p24 antigen [Ag]-positive cells) was determined by the standard ^{51}Cr release assay as described previously (42). The infected cells were incubated with 150 μCi $Na_2{}^{51}CrO_4$ in saline for 60 min, and then the infected cells were washed three times with RPMI 1640 medium containing 10% NCS. Labeled target cells (2×10^3/well) were added to each well of a U-bottom 96-well microtiter plate (Nunc, Roskilde, Denmark) with effector cells at an effector-to-target cell (E:T) ratio of 2:1. The cells were then incubated for 6 h at 37°C. The supernatants were collected and analyzed with a gamma counter.

Assay for suppression of HIV-1 replication by HIV-1-specific CTLs. The ability of HIV-1-specific CTLs to suppress HIV-1 replication was examined as previously described (42). CD4$^+$ T cells isolated from PBMCs were derived from an HIV-1-seronegative individual with HLA-B*5101. After the CD4$^+$ T cells had been incubated with the desired HIV-1 clones for 4 h at 37°C, they were washed three times with R10 medium. The HIV-1-infected CD4$^+$ T cells were then cocultured with HIV-1-specific CTL clones. From day 3 to day 7 postinfection, culture supernatants were collected, and the concentration of p24 Ag in the supernatants was measured by an enzyme-linked immunosorbent assay (ELISA) (HIV-1 p24 Ag ELISA kit; ZeptoMetrix).

Sequencing of proviral DNA or plasma RNA. Genomic DNA was extracted from PBMCs by using a QIAamp DNA blood minikit (Qiagen). Viral RNA was extracted from the plasma of HIV-1-infected individuals by using a QIAamp Mini Elute virus spin kit (Qiagen). cDNA was synthesized from the RNA with SuperScript II and random primers (Invitrogen). We amplified HIV RT and integrase sequences by nested PCR using RT-specific primers 5′-CCAAAAGT TAAGCAATGGCC-3′ and 5′-CCCATCCAAAGGAATGGAGG-3′ or 5′-CC TTGCCCCTGCTTCTGTAT-3′ for the first round of PCR and 5′-AGTTAGG AATACCACACCCC-3′ and 5′-GTAAATCCCCACCTCAACAG-3′ or 5′-AA TCCCCACCTCAACAGAAG-3′ for the second round and integrase-specific primers 5′-ATCTAGCTTTGCAGGATTCGGG-3′ and 5′-CCTTAACCGTAG TACTGGTG-3′ or 5′-CCTGATCTCTTACCTGTCC-3′ for the first round of PCR and 5′-AAAGGTCTACCTGGCATGGG-3′ or 5′-TTGGAGAGCAATG GCTAGTG-3′ and 5′-AGTCTACTTGTCCATGCATGGC-3′ for the second round. PCR products were either sequenced directly or cloned by using a TOPO TA cloning kit (Invitrogen) and then sequenced. Sequencing was done with a BigDye Terminator cycle sequencing kit (version 1.1; Applied Biosystems), and sequences were analyzed by use of an ABI PRISM 310 genetic analyzer.

Cell surface staining and intracellular cytokine staining (ICC assay). PBMCs from HIV-1-infected individuals were stimulated with the desired peptide (1 μM) and cultured for 12 to 14 days. These cultured PBMCs were assessed for gamma interferon (IFN-γ)-producing activity as previously described (42). After C1R-B*5101 cells had been incubated for 60 min with epitope peptides (1 μM), they were washed twice with RPMI 1640 containing 10% FCS. These C1R cells and the cultured PBMCs were incubated at 37°C for 6 h at an effector-to-stimulator ratio of 2:1 or 4:1 after the addition of brefeldin A (10 μg/ml). Next, the cells were stained with an anti-CD8 MAb (Dako Corporation, Glostrup, Denmark), fixed with 4% paraformaldehyde at 4°C for 20 min, and then permeabilized at 4°C for 10 min with PBS supplemented with 0.1% saponin containing 20% NCS (permeabilizing buffer). The cells were resuspended in the permeabilizing buffer and were then stained with an anti-IFN-γ MAb (BD Bioscience Pharmingen, San Diego, CA). Finally, they were resuspended in PBS containing 2% paraformaldehyde, and then the percentage of CD8$^+$ cells positive for intracellular IFN-γ was determined by using a FACSCalibur flow cytometer.

RESULTS

Association of HLA-B*5101 with long-term control of HIV-1 in HIV-1-infected Japanese hemophiliacs. We recruited 108 Japanese hemophiliacs who had been infected with HIV-1 before 1985. Eighteen of the patients had not been treated with any antiretroviral therapy (ART) and had CD4 counts of >350 (very-slow-progressor [VSP] group) by 1998, whereas the other 90 patients had been treated with ART and/or had a CD4 count of <350 (slow-progressor [SP] group). The frequency of HLA-B*5101 in the VSP group (9 of 18 donors [50.0%]) was higher than that in the SP group (15 of 90 donors [16.7%]), and the difference between these 2 groups was significant (P, 0.01). We analyzed the association of HLA class I alleles with disease progression during the years 1998 to 2007 in the VSP group. The 9 HLA-B*5101$^+$ VSP hemophiliacs exhibited significantly slower progression of the disease over this period than the 9 HLA-B*5101$^-$ subjects (Fig. 1), and no other HLA-B alleles or HLA-A/DR alleles showed any significant influence on the progression of the disease in this group (not shown). One HLA-B*3501$^+$ VSP hemophiliac was found in the HLA-B*5101$^+$ group, but none were found in the HLA-B*5101$^-$ group, indicating that HLA-B*3501, which is associated with rapid progression to AIDS, did not affect the results for the 2 VSP groups. Other HLA-A/B/DR alleles were not associated with the HLA-B*5101$^+$ or the HLA-B*5101$^-$ group (see Table S1 in the supplemental material). These results, taken together, show that the HLA-B*5101 allele was

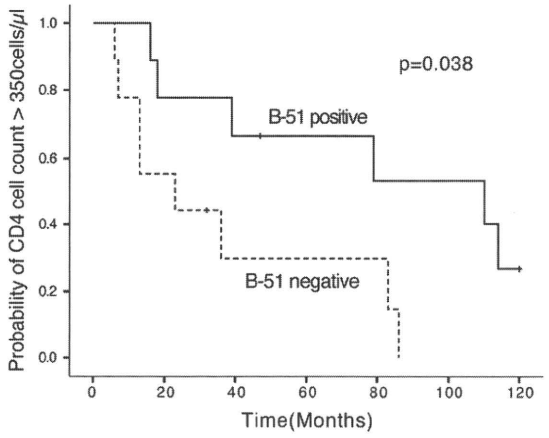

FIG. 1. Association of HLA-B*5101 with slow progression to AIDS. Kaplan-Meier survival analysis was used to estimate the time to the first CD4 cell count (24-week time-weighted average levels of CD4 cells) of $<350/\mu l^3$ for 9 HLA-B*5101-positive (solid line) and 9 HLA-B*5101-negative (dashed line) hemophiliacs who had not been treated with antiretroviral therapy (ART) and who had a CD4 count of $>350/\mu l$ in 1998.

still associated with slow progression of the disease more than 20 years postinfection.

Control of HIV-1 replication by HLA-B*5101-restricted CD8$^+$ T cells. A previous study demonstrated that 2 types of HLA-B*5101-restricted CTLs, Pol283-8 (TAFTIPSI)-specific and Pol743-9 (LPPVVAKEI)-specific CTLs, suppressed HIV-1 replication *in vitro* much more strongly than did other HLA-B*5101-restricted CTLs (43), suggesting that these CTLs may play a key role in the control of HIV-1 in the HLA-B*5101$^+$ SP group. To investigate the control of HIV-1 by these CTLs, we selected 10 HLA-B*5101-positive donors (8 VSPs and 2 SPs) who had not been treated with ART by 1998 and whose PBMC samples were available for analysis of HLA-B*5101-restricted CTLs (see Fig. S1 and Table S2 in the supplemental material). Three of the 8 VSP patients had VLs below 1,000 copies at all time points tested and were classified as LTNPs. We found that only 3 of the 108 HIV-1-infected hemophiliacs (KI-021, KI-051, and KI-124) were LTNPs for approximately 25 years and that all 3 of these LTNPs carried HLA-B*5101. We generated 4 HLA-B*5101 tetramers carrying Pol283-8, Pol743-9, Gag327-9, or Rev71-11, and we used them to determine the frequencies of HIV-1-specific CD8$^+$ T cells among PBMCs from these 3 LTNPs (Table 1 and Fig. 2). KI-021 had both Pol283-8- and Pol743-9-specific CD8$^+$ T cells but neither Gag327-9- nor Rev71-11-specific CD8$^+$ T cells during the years 1997 to 2005 (Fig. 2A). KI-051 also had both Pol283-8- and Pol743-9-specific CD8$^+$ T cells, whereas this patient had no Rev71-11-specific CD8$^+$ T cells and a low number of Gag327-9-specific CD8$^+$ T cells during the years 1999 to 2005 (Fig. 2B). KI-124 had Pol283-8-, Pol743-9-, and Gag327-9-specific CD8$^+$ T cells (Table 1). These results suggest that the 2 Pol-specific CD8$^+$ T cells may play an important role in the control of HIV-1 in these LTNPs carrying HLA-B*5101.

Selection of escape mutations of the Pol283-8 epitope in very slow progressors. Of the 8 HLA-B*5101$^+$ VSP hemophiliacs, KI-127 had Pol283-8-specific CD8$^+$ T cells at a low frequency in 1998, when the plasma viral load (pVL) was very low, whereas later this patient lost the response, and the pVL increased from an undetectable level to more than 10^3 copies (Fig. 2C). The other 4 VSPs, excluding 3 LTNBs, either had a low number of Pol283-8-specific CD8$^+$ T cells or did not have any of these cells at any time points studied. These results suggest that Pol283-8-specific CD8$^+$ T cells rather than Pol743-9-specific CD8$^+$ T cells may control HIV-1 *in vivo*.

To clarify the role of these HLA-B*5101-restricted CD8$^+$ T cells in the control of HIV-1 *in vivo*, we analyzed the correlation between the frequency of the HLA-B*5101-restricted CD8$^+$ T cells and the pVL in 10 HLA-B*5101$^+$ hemophiliacs. The frequency of Pol283-8-specific CD8$^+$ T cells was negatively correlated with the pVL (P, 5.6×10^{-8}), whereas the frequency of the other T cells was positively correlated with the pVL (Fig. 3). These results support the idea that Pol283-8-specific CD8$^+$ T cells drive the suppression of HIV-1 replication *in vivo*.

We speculated, therefore, that escape mutants within Pol283-8 epitopes were selected in slow progressors over a 25-year period, because these epitope-specific CTLs are thought to provide strong immune pressure on HIV-1. Two of the LTNPs had the Pol283-8V mutant, whereas the third had wild-type Pol283 in July 2002 but the 8V mutant in October

TABLE 1. Numbers of 4 types of HLA-B*5101-restricted CD8$^+$ T cells among HLA-B*5101$^+$ HIV-1-infected hemophiliacs

Patient	Median VL (copies/ml)[a]	Median no. of CD4 cells/μl[a]	Median no. (frequency) of HLA-B*5101-restricted CD8$^+$ T cells[b]				No. of times PBMCs were tested (dates)[c]
			Pol743	Pol283	Gag327	Rev71	
KI-021	50	618	1,910 (0.39)	1,900 (0.40)	<100 (0)	<100 (0)	10 (8/1997–11/2005)
KI-051	50	737	3,222 (0.53)	5,186 (0.87)	1,082 (0.16)	<100 (0)	5 (10/1999–9/2005)
KI-124	570	850	3,126 (0.43)	1,745 (0.24)	1,381 (0.19)	<100 (0)	8/2001
KI-386	360	459	3,164 (0.40)	554 (0.07)	5,774 (0.73)	396 (0.05)	8/2006
KI-363	1,700	676	6,696 (0.54)	1,488 (0.12)	496 (0.04)	1,116 (0.09)	11/1998
KI-127	5,500	597	8,100 (0.79)	257 (0.02)	23,411 (2.33)	<100 (0.01)	9 (2/1998–4/2006)
KI-121	16,650	327	4,853 (0.59)	134 (0.02)	<100 (0)	395 (0.04)	2 (12/1999, 8/2001)
KI-032	25,500	226	9,153 (1.80)	<100 (0)	344 (0.09)	<100 (0)	2 (10/2002, 9/2005)
KI-007	39,500	387	1,084 (0.12)	394 (0.05)	6,278 (0.68)	1,029 (0.12)	2 (6/2001, 4/2002)
KI-026	40,000	526	10,705 (1.32)	<100 (0)	6,164 (0.76)	568 (0.07)	7/2005

[a] At the time of tetramer analysis.
[b] Median number of HLA-B*5101-restricted CD8$^+$ T cells/μl among PBMCs (median frequency of HLA-B*5101-restricted T cells among CD8$^+$ T cells [expressed as a percentage]).
[c] If PBMCs were tested only once, only the date (month/year) is given.

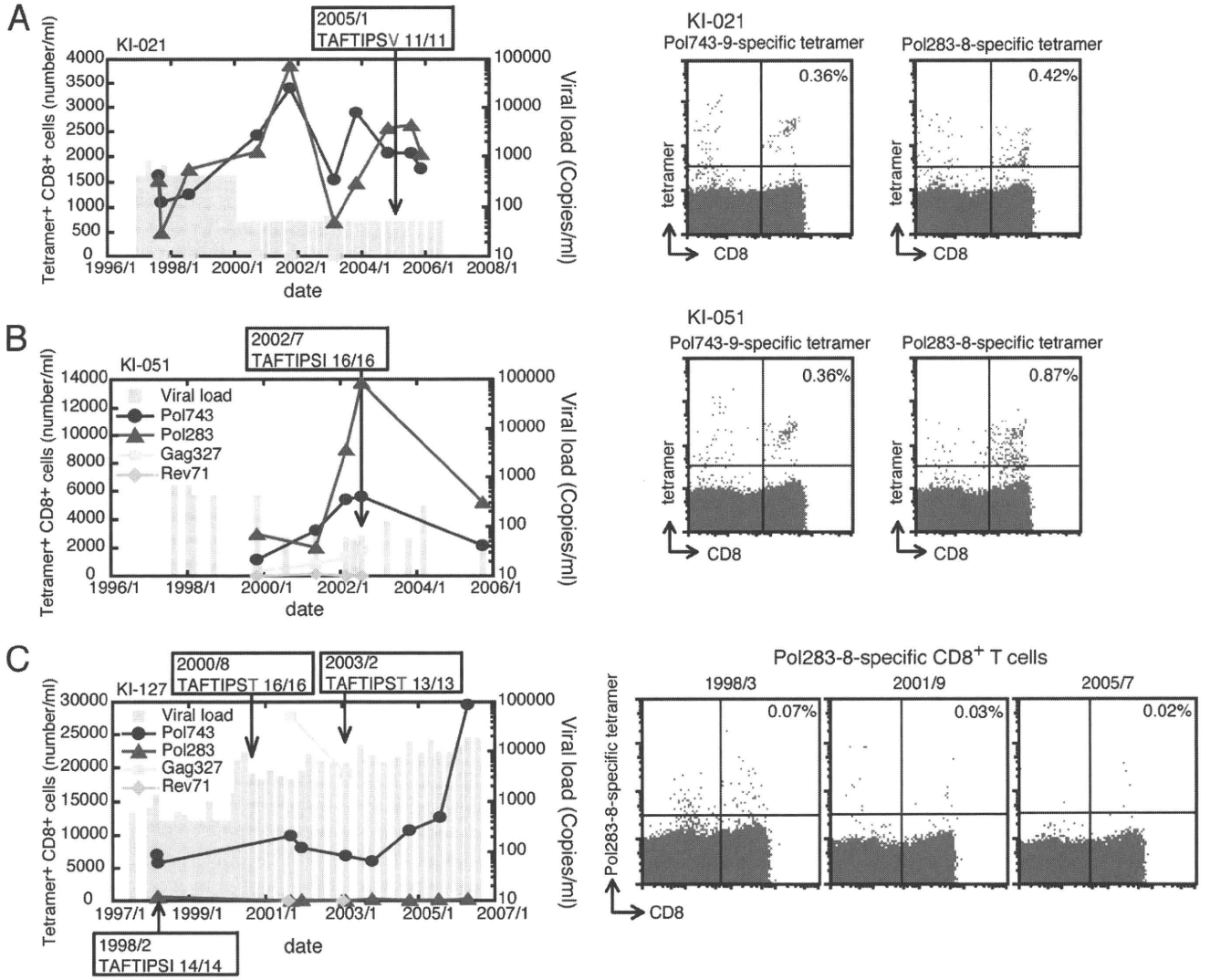

FIG. 2. Longitudinal analysis of HLA-B*5101-restricted $CD8^+$ T cells and Pol283 epitope sequences in 3 slow-progressing hemophiliacs. Four types of HIV-1-specific $CD8^+$ T cells were detected by use of specific tetramers. PBMCs from KI-021 (A), KI-051 (B), and KI-127 (C) were analyzed by using Pol743-9-specific and Pol283-8-specific tetramers. The percentage of tetramer-positive cells among the $CD8^+$ T-cell population is given in the upper right quadrant of each histogram. The sequence of the Pol283-8 epitope from each patient is shown. The detection limit of pVL was 400 copies/ml until 2000 and 50 copies/ml after 2000.

2006 (Table 2). As previously noted (34), Pol283-8-specific CTL clones showed the same killing activity toward target cells prepulsed with the Pol283-8V peptide as toward those prepulsed with the wild-type peptide. These T cells revealed similar killing activity toward 721.221-B*5101 cells infected with NL-432 carrying Pol283-8V (NL-Pol283-8V) as toward those infected with NL-432 (see Fig. S2A in the supplemental material) and only a marginally weaker ability to suppress the replication of NL-Pol283-8V (see Fig. S2B in the supplemental material). In contrast, the 5 VSPs and 2 SPs had Pol283-8T or Pol283-8R mutants (Table 2). Three Pol283-8-specific CTL clones failed to kill target cells infected with NL-432 carrying these mutants (NL-Pol283-8T and NL-Pol283-8R [see Fig. S2A in the supplemental material]) or to suppress the replication of these mutants (see Fig. S2B in the supplemental material), indicating that these were escape mutants.

Longitudinal analysis of KI-127 showed that the 8T mutant appeared in August 2000, when the VL had increased approximately 10-fold, whereas wild-type Pol283 was found in February 1998, when the VL was very low or undetectable (Fig. 2C). Previous population analysis using 9 cohorts showed strong association between HLA-B*51 and Pol283-8T (20). These observations together suggest that the 8T mutant is an escape mutant selected by Pol283-specific CTLs and implies that escape from this epitope reduces immune control of HIV-1.

***In vitro* selection of Pol283 escape mutants by Pol283-specific CTLs.** The results shown in Fig. 4 suggested that Pol283-specific CTLs selected 8T, 8R, and 8L escape mutants. To further confirm the selection of these mutants by Pol283-specific CTLs, we investigated whether Pol283-specific CTLs selected these mutant viruses *in vitro* when the CTLs were cul-

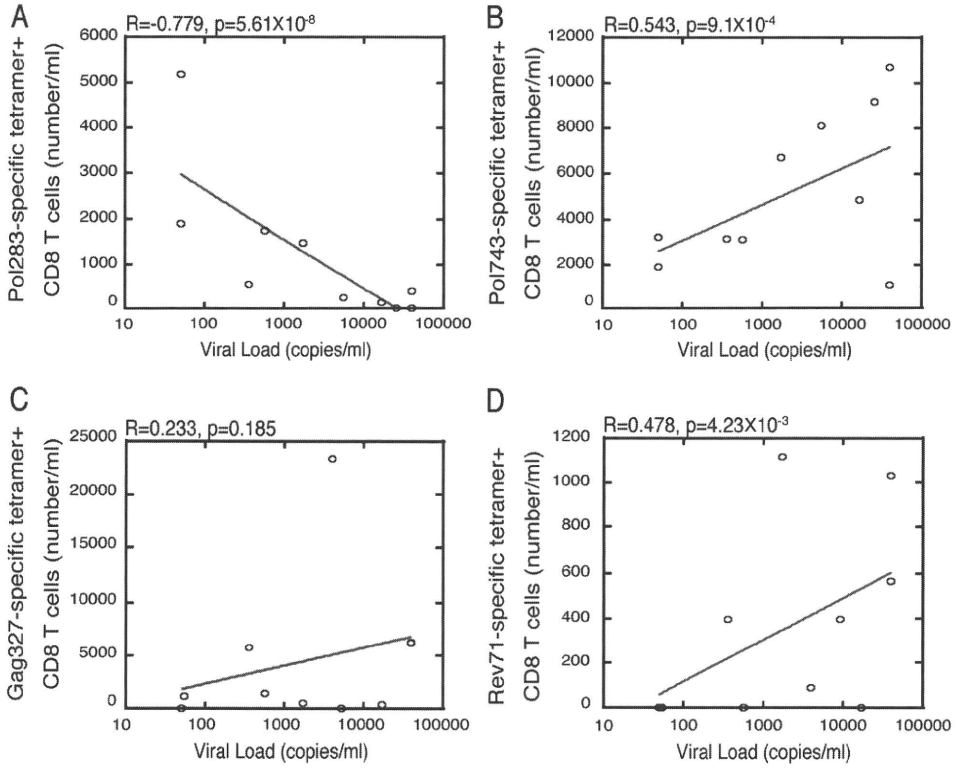

FIG. 3. Correlation of the number of HLA-B*5101-restricted CD8+ T cells with the viral load. The number of Pol283-8-specific (A), Pol743-9-specific (B), Gag327-specific (C), or Rev71-specific (D) CD8+ T cells among PBMCs from 10 HLA-B*5101+ hemophiliacs was measured at 1 time point or at 2 to 10 different time points (see Table 1) by using specific tetramers. The correlation of the median number of tetramer-positive cells with the median viral load was analyzed.

tured with HLA-B*5101-positive CD4+ T cells infected with NL-432 and the mutant virus together. Pol283-specific CTL clones selected these 3 mutant (8T, 8R, and 8L) viruses rapidly in this assay (Fig. 4A to C), supporting the notion that these mutants were selected as escape mutants by Pol283-specific CTLs.

Long-term maintenance of Pol283-8-specific memory CD8+ T cells and failure of induction of escape mutant-specific CD8+ T cells. If the Pol283-8T mutant was selected by Pol283-8-specific CTLs in donors first infected with HIV-1 carrying the Pol283-8 wild-type epitope, we can speculate that the donors had Pol283-8-specific memory CD8+ T cells but failed to elicit

TABLE 2. Sequences of Pol283-8 and Pol743-9 epitopes in HLA-B*5101+ HIV-1-infected hemophiliacs

Patient	Epitope				VL (copies/ml)	Date (mo/yr) of PBMC testing[b]
	Pol283-8		Pol743-9			
	Sequence	Clonal frequency[a]	Sequence	Clonal frequency		
NA[c] (wild-type sequence)	TAFTIPSI		LPPVVAKEI			
KI-021	-------V	11/11	---------	10/12	<50	1/2005
KI-051	---------	16/16	---------	15/15	63	7/2002
	-------V	DS	ND[d]	ND	<50	10/2006
KI-124	-------V	14/14	---------	14/15	600	8/2001
KI-386	-------T	DS	---------	DS	1,200	10/2006
KI-363	-------T	DS	---------	DS	1,700	11/1998
KI-127	-------T	13/13	---------	17/17	5,300	2/2003
KI-121	-------T	16/16	I--------	12/13	9,300	12/1999
KI-032	-------T	13/13	---------	15/15	17,000	10/2002
KI-007	-------R	15/16	---II----	18/18	33,000	6/2001
KI-026	-------T	DS	I--------	DS	28,000	1/2004

[a] Expressed as (number of clones carrying the indicated sequence)/(number of clones tested). DS, direct sequence.
[b] The sequence for patient KI-021 is from proviral DNA; those for all other patients are from plasma RNA.
[c] NA, not applicable.
[d] ND, not determined.

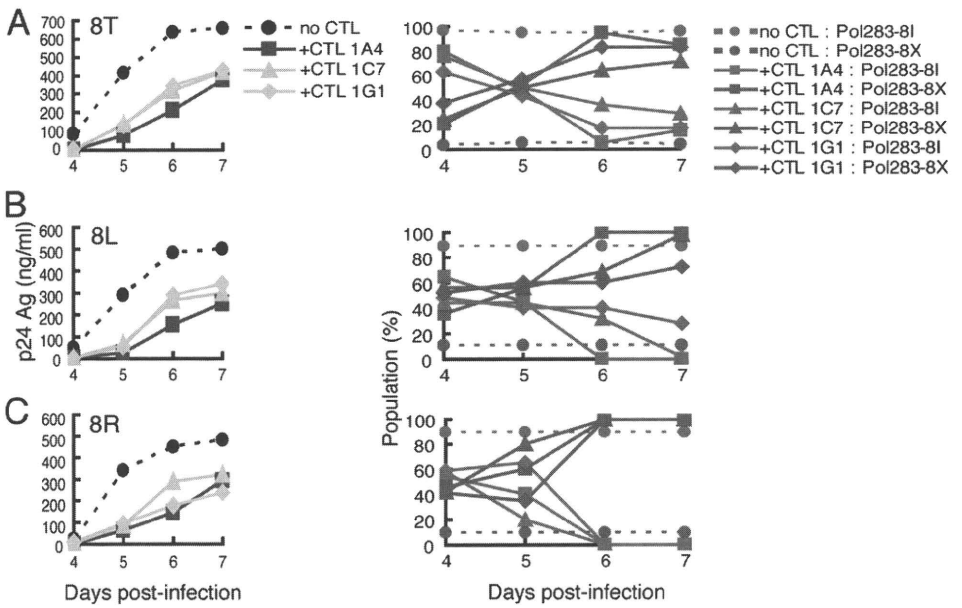

FIG. 4. *In vitro* selection of Pol283 escape mutants by a Pol283-8-specific CTL clone. T1 cells were infected with paired viruses (NL-432 [Pol283-8I] and a mutant virus [Pol283-8L, -8T, or -8R]) at a ratio of 9:1. The infected cells were incubated with Pol283-8-specific CTL clones at an E:T ratio of 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

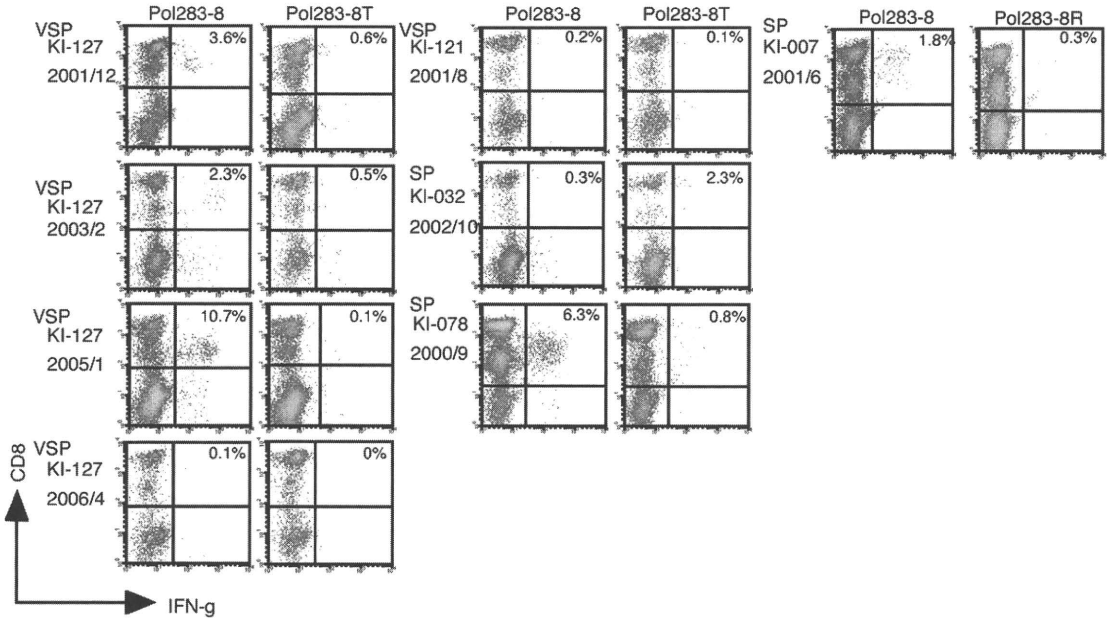

FIG. 5. Induction of Pol283-8-specific CD8+ T cells from PBMCs of 2 very slow progressors and 3 slow progressors. PBMCs from 2 very slow progressors (KI-127 and KI-121) and from 3 slow progressors (KI-032, KI-007, and KI-078) were stimulated with the Pol283-8 epitope peptide or the Pol283-8T or -8R peptide 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-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-B^*5101^+ individuals initially infected with the mutant virus. We showed previously that recently infected HLA-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.

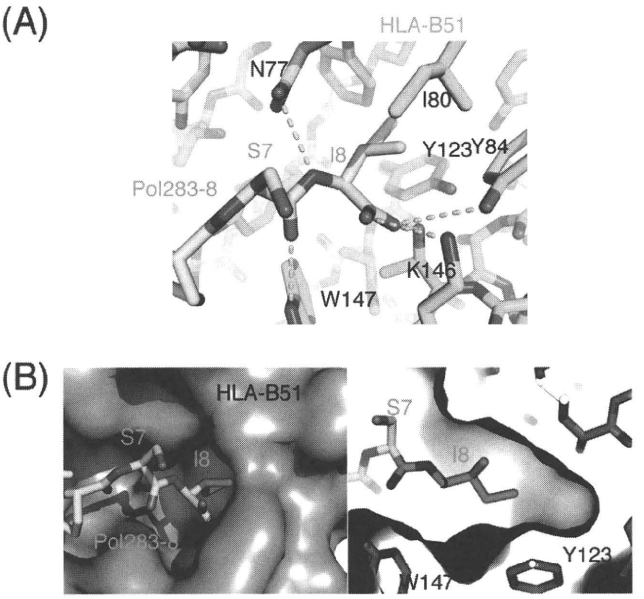

FIG. 6. Binding model of HLA-B*5101 mutant peptides. (A) Polar interactions around the PC residue in the HLA-B51–Pol283-8 complex. The Pol283-8 peptide and the HLA-B51 heavy chain are shown as yellow and cyan stick models, respectively (N and O atoms are shown as blue and red, respectively). The dotted 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-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-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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Trends in transmitted drug-resistant HIV-1 and demographic characteristics of newly diagnosed patients: Nationwide surveillance from 2003 to 2008 in Japan

Junko Hattori[a], Teiichiro Shiino[b], Hiroyuki Gatanaga[c], Shigeru Yoshida[d], Dai Watanabe[e], Rumi Minami[f], Kenji Sadamasu[g], Makiko Kondo[h], Haruyo Mori[i], Mikio Ueda[j], Masao Tateyama[k], Atsuhisa Ueda[l], Shingo Kato[m], Toshihiro Ito[n], Masayasu Oie[o], Noboru Takata[p], Tsunefusa Hayashida[c], Mami Nagashima[g], Masakazu Matsuda[q], Shiro Ibe[a], Yasuo Ota[r], Satoru Sasaki[n], Yoshiaki Ishigatsubo[l], Yoshinari Tanabe[o], Ichiro Koga[r], Yoko Kojima[i], Masahiro Yamamoto[f], Jiro Fujita[k], Yoshiyuki Yokomaku[a], Takao Koike[s], Takuma Shirasaka[e], Shinichi Oka[c], Wataru Sugiura[a,b,t,*]

[a] National Hospital Organization, Nagoya Medical Center, Clinical Research Center, 4-1-1 Sannomaru, Naka-ku, Nagoya 4600001, Japan
[b] National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 1628640, Japan
[c] AIDS Clinical Center, National Center for Global Health and Medicine, 1-21-1 Toyama, Shinjuku-ku, Tokyo 1628655, Japan
[d] Faculty of Health Sciences, Hokkaido University, N12-W5, Kita-ku, Sapporo 0600812, Japan
[e] National Hospital Organization, Osaka Medical Center, 2-1-14 Houenzaka, Chuo-ku, Osaka 5400006, Japan
[f] National Hospital Organization, Kyushu Medical Center, 1-8-1 Jigyohama, Chuo-ku, Fukuoka 8108563, Japan
[g] Tokyo Metropolitan Institute of Public Health, 3-24-1 Hyakunin-cho, Shinjuku-ku, Tokyo 1690073, Japan
[h] Kanagawa Prefectural Institute of Public Health, 1-3-1 Shimomachiya, Chigasaki, Kanagawa 2530087, Japan
[i] Osaka Prefectural Institute of Public Health, 1-3-69 Nakamichi, Higashinari-ku, Osaka 5370025, Japan
[j] Ishikawa Prefectural Central Hospital, 2-1 Anngetsu-higashi, Kanazawa 9208530, Japan
[k] University of the Ryukyus, 207 Uehara, Nishihara, Okinawa 9030125, Japan
[l] Yokohama City University Graduate School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama 2360004, Japan
[m] Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 1608582, Japan
[n] National Hospital Organization, Sendai Medical Center, 2-8-8 Miyagino, Miyagino-ku, Sendai 9830045, Japan
[o] Niigata University Graduate School of Medical and Dental Sciences, 1-757 Asahimachi-dori, Chuo-ku, Niigata 9518510, Japan
[p] Hiroshima University Hospital, 1-2-3 Kasumi, Minami-ku, Hiroshima 7348551, Japan
[q] Mitsubishi Chemical Medience Corporation, 3-30-1 Shimura, Itabashi-ku, Tokyo 1748555, Japan
[r] Teikyo University School of Medicine, 2-11-1 Kaga, Itabashi-ku, Tokyo 1738605, Japan
[s] Hokkaido University Graduate School of Medicine, N15-W7, Kita-ku, Sapporo 0608638, Japan
[t] Nagoya University Graduate School of Medicine, 65 Tsurumai, Showa-ku, Nagoya 4668550, Japan

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ABSTRACT

The emergence and transmission of drug-resistant human immunodeficiency virus-1 (HIV-1) compromises antiretroviral treatment for HIV-1. Thus, testing for drug resistance is recommended at diagnosis and before initiating highly active antiretroviral treatment. We conducted an epidemiological study enrolling newly diagnosed patients between 2003 and 2008 in our nationwide surveillance network. In the 6-year study period, the prevalence of drug-resistant HIV-1 among 2573 patients, consisting mainly of Japanese men in their late-30s and infected through male-to-male sexual contacts, followed an increasing trend from 5.9% (16/273) in 2003 to 8.3% (50/605) in 2008. Nucleoside reverse transcriptase inhibitor-associated mutations predominated in each year, with T215 revertants being the most abundant. The predictive factor for drug-resistant HIV-1 transmission was subtype B (OR = 2.36; $p = 0.004$), and those for recent HIV-1 infection were male gender (OR = 3.79; $p = 0.009$), MSM behavior (OR = 1.67; $p = 0.01$), Japanese nationality (OR = 2.31; $p = 0.008$), and subtype B (OR = 5.64; $p < 0.05$). Continued activities are needed to raise awareness of the risks of HIV-1 infection and complications of drug-resistant strains. Continued surveillance is also needed to understand trends in the HIV-1 epidemic.

Abbreviations: HIV-1, human immunodeficiency virus type 1; HAART, highly active antiretroviral therapy; PI, protease inhibitor; HBV, hepatitis B virus; HCV, hepatitis C virus; PR, protease; RT, reverse transcriptase; RT-PCR, reverse transcription polymerase chain reaction; CRF, circulating recombinant form; NRTI, nucleoside RT inhibitor; NNRTI, non-nucleoside RT inhibitor; OR, odds ratio; CI, confidence interval; MSM, men who have sex with men; IDU, intravenous drug user.

* Corresponding author at: Clinical Research Center, National Nagoya Medical Center, 4-1-1 Sannomaru Naka-ku, Nagoya, Aichi 4600001, Japan. Tel.: +81 52 951 1111; fax: +81 52 963 3970.
E-mail address: wsugiura@nih.go.jp (W. Sugiura).

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1. Introduction

The emergence of drug-resistant human immunodeficiency virus type 1 (HIV-1) among patients under highly active antiretroviral therapy (HAART) limits the successful suppression of HIV-1 replication. Several years after the introduction of HAART, drug-resistant strains are being detected among newly diagnosed HAART-naïve patients, suggesting the transmission of drug-resistant HIV-1 from the treatment-exposed population. Thus, treatment-naïve patients have been recommended by the US Department of Health and Human Services, International AIDS Society-USA, and other drug-resistance testing guidelines to undergo drug resistance testing at diagnosis and before initiation of HAART (DHHS, 2009; Hirsch et al., 2000, 2008). Indeed, choosing effective antiretrovirals according to the results obtained from this testing has led to successful control of HIV-1 infection. Furthermore, the drug resistance testing at diagnosis helps to understand transmission of drug-resistant HIV-1 in HAART-naïve individuals which in turn may help prevent transmission events.

The prevalence of drug-resistant HIV-1 among treatment-naïve patients has been closely monitored and reported from many countries. Before and early in the HAART era, when only mono or dual therapy was available, the prevalence was as high as 10–20% (Boden et al., 1999; Gómez-Cano et al., 1998; Tambussi et al., 1998). However, after the introduction of antiretrovirals with better pharmacokinetics, such as ritonavir-boosted protease inhibitor (PI), the emergence of drug-resistant viruses seemed to decrease (Gallego et al., 2001; Maia Teixeira et al., 2006).

Furthermore, despite the great number of HIV-1-infected patients, the prevalence tended to be low in developing countries where patients had limited or no access to antiretroviral drugs, e.g., 0–4.2% in Africa (Bártolo et al., 2009; Mintsa-Ndong et al., 2009; Ndembi et al., 2008; Pillay et al., 2008), 1.5% in Cambodia (Nouhin et al., 2009), and 2.6% in Vietnam (Ishizaki et al., 2009). In contrast, in countries where antiretroviral drugs are more accessible, the prevalence has been higher, e.g., 5.2% in Thailand (Apisarnthanarak et al., 2008), 9.4% in Taiwan (Chang et al., 2008), 10.0% in India (Lall et al., 2008), 7.8% in Portugal (Palma et al., 2007), 9.0% in Germany (Sagir et al., 2007), 9.5% in Belgium (Vercauteren et al., 2008), 10.9% in France (Chaix et al., 2009), and 15.9% in the US (Eshleman et al., 2007).

In Japan, since the first HIV-1-infected case was identified in 1985, the annual number of reported cases has been increasing every year, reaching 15 451 by the end of 2008. With more people getting infected, larger numbers of patients are starting anti-HIV-1 treatment and the risk of emerging drug-resistant HIV-1 is increasing. To understand the trends in drug-resistant HIV-1 in Japan, a nationwide surveillance project has been in effect since 2003. In our previous report of surveillance results from 2003 to 2004, the prevalence of drug-resistant HIV-1 in newly diagnosed patients was 4.0% (Gatanaga et al., 2007). We have continued collecting and analyzing data from newly diagnosed HIV-1-infected patients at participating clinical and research facilities in Japan. We report here the prevalence of drug-resistant HIV-1 among newly diagnosed therapy-naïve patients between 2003 and 2008.

2. Materials and methods

2.1. Sample

The study population included all the HIV-1-infected patients newly diagnosed between January 2003 and December 2008 at any of the participating HIV/AIDS clinics. Drug resistance genotypic tests were performed at 12 laboratories including 8 clinical laboratories at HIV/AIDS clinics, 3 public health laboratories, and the National Institute of Infectious Diseases. After patients agreed to participate in our surveillance project and gave informed consent, peripheral blood was drawn with EDTA added, and their demographic and clinical information were collected. Demographic information included age, gender, nationality, and risk behavior. Clinical data included HIV-1 viral loads, $CD4^+$ T cell counts, status of hepatitis B and C virus (HBV, HCV) co-infection, baseline sequence data, and drug-resistant amino acid mutations.

This study was conducted according to the principles in the Declaration of Helsinki, and was approved by the ethical committee of the National Institute of Infectious Diseases, Japan. By Japanese law, HIV-1-infected patients must be reported to the Japanese Ministry of Health, Labour, and Welfare upon diagnosis. The numbers reported to the Ministry are considered the "official numbers" of newly diagnosed HIV/AIDS cases, and were used as comparison controls to evaluate our study population.

2.2. Drug resistance genotypic testing

Drug resistance genotypic testing was performed using in-house protocols. Briefly, viral RNA was extracted from patient plasma samples. HIV-1 protease (PR, 1–99 amino acids) and the N-terminal region of reverse transcriptase (RT, 1–240 amino acids) were amplified in reverse transcription polymerase chain reaction (RT-PCR) followed by nested PCR using in-house primer sets. Subsequently, the amplified PCR products were purified and their sequences were analyzed by direct sequencing method using an automated sequencer. The resulting electropherograms were analyzed using commercially available software. The quality of testing methods used at each participating facility was assessed and confirmed for detection of drug-resistant mutations (Fujisaki et al., 2007). Thus, detection of drug-resistant mutations was consistent among facilities.

2.3. Determination of HIV-1 subtypes and drug-resistant HIV-1

HIV-1 subtypes were determined using the sequences of HIV-1 PR and RT genes obtained in the drug resistance genotypic testing explained above. Each sequence was aligned with the reference sequences of HIV-1 subtypes A through K, and circulating recombinant forms (CRFs), all of which were obtained from the Los Alamos HIV Databases (Los Alamos, 2010), using ClustalW, and phylogenetic trees were constructed using the neighbor-joining method with bootstrap value of 1000.

The resulting sequences were compared to that of HXB2 to judge the presence of amino acid mutations. The drug-resistant mutations were determined according to criteria of the HIV Drug Resistance Database of Stanford University (Bennett et al., 2009). Thus, a sample was considered to harbor drug-resistant HIV-1 if it possessed any of the following mutations: in the PR gene, L23I, L24I, D30N, V32I, M46I/L, I47V/A, G48V/M, I50V/L, F53L/Y, I54V/L/M/A/T/S, G73S/T/C/A, L76V, V82A/T/F/S/C/M/L, N83D, I84V/A/C, I85V, N88D/S, and L90M (indicating PI resistance); in the RT gene, M41L, K65R, D67N/G/E, T69D/insertion, K70R/E, L74V/I, V75M/T/A/S, F77L, Y115F, F116Y, Q151M, M184V/I, L210W, T215Y/F/I/S/C/D/V/E, K219Q/E/N/R (indicating nucleoside RT inhibitor [NRTI] resistance), and L100I, K101E/P, K103N/S, V106M/A, V179F, Y181C/I/V, Y188L/H/C, G190A/S/E, P225H, M230L (indicating non-nucleoside RT inhibitor [NNRTI] resistance).

2.4. BED assay

The time of HIV-1 seroconversion was estimated in randomly selected samples as recent (within 155 days) or not recent using the BED assay (Calypte HIV-1 BED Incidence EIA, BioRad) according to the Manufacturer's instruction. Briefly, 5 μL of plasma was diluted

with 500 μL of sample diluent in the kit, and the proportion of anti-HIV-1 IgG to a total IgG in the sample was measured by optical density.

2.5. Statistical analysis

Statistical analyses were performed using R software (SAS Institute). Chi-square or Fisher's exact probability tests were used to determine associations among patients' demographic characteristics, nationality, BED assay results, and transmission of drug resistance. The odds ratio (OR) and 95% confidence intervals (CI) were calculated for all the variables. Recent and not-recent seroconversion groups were examined for differences in HIV-1 viral loads by analysis of covariance (ANCOVA), with $CD4^+$ T cell count as the covariate.

3. Results

3.1. Majority of treatment-naïve patients are Japanese men who have sex with men (MSM) in mid-30s

The demographics of the 2573 newly diagnosed HIV-1-infected patients enrolled between 2003 and 2008 are summarized in Table 1. Male ($n = 2397$, 93.2%), Japanese (90.1%), and those infected through male-to-male sexual contact (68.9%) predominated, and the median age was 35. For the female cases ($n = 170$), high-risk heterosexual contact was the major risk factor ($n = 152$, 89.4%), and approximately half were non-Japanese ($n = 63$, 41.4%). Further analysis showed a significant association between the transmission route and nationality, i.e., most Japanese patients were infected through male-to-male sexual contact, while non-Japanese patients were infected by other routes (OR = 5.60; 95% CI 4.14–7.63; $p < 0.01$) (Table 2). It should be noted that sexual contacts (92.1%) are the major risk factor for HIV-1 infection in Japan. On the other hand, injecting drug usage, one of the high risk factors in other countries, accounts for only 0.4%.

HBV and/or HCV co-infection, an important clinical factor affecting prognosis and treatment of HIV infection (Ockenga et al., 1997; Piroth et al., 2000), was found to have a prevalence of 8.4% of 2101 patients, and 4.7% of 2071, respectively (Table 1). These prevalence rates did not change significantly throughout the study period (supplementary Table 1). HBV co-infection was found to be significantly associated with subtype B (OR = 2.04; $p < 0.05$) or infection through male-to-male sexual contact (OR = 1.66; $p < 0.05$).

3.2. Subtype B HIV-1 predominates in Japan

Of 2573 plasma samples collected during the study period, the sequences of PR and RT genes were successfully amplified and analyzed in 2536 (98.6%) and 2534 (98.5%) samples, respectively. Of these, we examined sequences of the PR-RT region from 2496 cases by phylogenetic tree analysis to determine the distribution of HIV-1 subtypes in Japan. Subtype B HIV-1 was found to predominate among the study population ($n = 2194$, 87.9%). The remaining non-B subtypes included 210 (8.4%) CRF01_AE, 30 (1.2%) C, 19 (0.8%) CRF02_AG, 18 (0.7%) A, 9 (0.4%) G, 7 (0.3%) F, 5 (0.2%) D, and 1 (0.04%) CRF08_BC (Table 1). In addition, 1 recombinant case of K/C, A/K, and D/B was detected in 2005, 2006, and 2007, respectively. These non-B subtype viruses were found mostly among the heterosexually infected population (223/302, 73.8%). In contrast, subtype B HIV-1 was found in the vast majority of MSM (1700/1773, 95.9%). In terms of nationality, Japanese patients, most of whom were MSM, were infected with subtype B HIV-1. On the other hand, only about a half of non-Japanese patients harbored subtype B HIV-1, and the remaining half were infected with non-B HIV-1, such as CRF01_AE (OR = 8.85; 95% CI 6.46–12.1; $p < 0.01$) (Table 2). This result is reasonable considering that the predominant HIV-1 subtype differs by country, and our study population included many Thais and Malaysians. In addition, this result suggests that subtype B HIV-1 is transmitted in a closed community of MSM, while non-B subtype strains are spread in wider areas among those infected through high-risk heterosexual contacts.

Table 1
Demographic characteristics of newly diagnosed HIV/AIDS patients.

	6-Year total (2573)	
Age		
Average	37.4	
Median	35	
Mode	35	
Quartile (Q1, Q3)	29, 43	
Nationality	*n*	(%)
Japanese	2319	(90.1)
Non-Japanese	225	(8.7)
Asian	83	(3.2)
Oceanian	4	(0.2)
North American	17	(0.7)
South American	58	(2.3)
European	10	(0.4)
African	26	(1.0)
Unspecified[a]	27	(1.0)
Unknown	29	(1.1)
Transmission category		
Male	2397	(93.2)
Male-to-male sexual contact	1773	(68.9)
High-risk heterosexual contact	369	(14.3)
Sexual contact	75	(2.9)
IDU	8	(0.3)
Other[b]	26	(1.0)
Unidentified	146	(5.7)
Female	170	(6.6)
High-risk heterosexual contact	152	(5.9)
IDU	3	(0.1)
Other[b]	5	(0.2)
Unidentified	11	(0.4)
Unknown	6	(0.2)
Unidentified	6	(0.2)
Hepatitis co-infection[c]		
HBV		
(+)	176	(8.4)
(−)	1925	(91.6)
Unknown	472	
HCV		
(+)	98	(4.7)
(−)	1973	(95.3)
Unknown	502	
HIV-1 subtype[c]		
B	2194	(87.9)
non-B	302	(12.1)
AE	210	(8.4)
C	30	(1.2)
AG	19	(0.8)
A	18	(0.7)
G	9	(0.4)
F	7	(0.3)
D	5	(0.2)
Other	4	(0.2)
Unidentified	77	

[a] Unspecified individuals in the nationality category were identified only as of non-Japanese origin.
[b] Other transmission categories include mother-to-child, blood products, transfusion, and needle stick.
[c] Prevalence of subtypes, HBV, and HCV was calculated after omitting the unidentified or unknown data. DU, intravenous drug user; HBV, hepatitis B virus; HCV, hepatitis C virus; HIV-1, human immunodeficiency virus type 1.

3.3. Prevalence of drug-resistant HIV-1 is increasing in Japan

A total of 194 cases (7.7%) in the 6-year study period were found to harbor HIV-1 strains with at least one major drug-resistant muta-

Table 2
Characteristics of newly diagnosed Japanese and non-Japanese HIV/AIDS patients.

	Nationality (*n*)			Odds ratio
	Japanese	Non-Japanese	Unknown	
Gender				
Male	2224	151	22	11.45*
Female	95	74	1	
Unknown[b]			6	
Transmission category				
Male-to-male sexual contact	1691	73	9	5.60[a,*]
High-risk heterosexual contact	399	114	7	
Sexual contact	72	4	0	
Other	29	10	2	
Unidentified[b]	128	24	11	
Subtype				
B	2051	118	25	8.85*
Non-B	198	101	3	
Unidentified[b]	70	6	1	
BED assay (*n* = 640)				
Recent	220	13	0	2.31*
Not recent	351	48	8	
Drug-resistant HIV-1				
Detected	173	16	5	1.05
Not detected	2146	209	24	

[a] Odds ratios for the transmission category were calculated between male-to-male sexual contact and other categories which include high-risk heterosexual contact, sexual contact, and other.
[b] Unknown and Unidentified cases were omitted in calculation of odds ratio.
* $p < 0.01$.

tion conferred by PIs, NRTIs, or NNRTIs. The annual prevalence of drug-resistant mutations shown in Fig. 1 had an overall tendency to increase from 5.9% (16/273) in 2003 to 8.3% (50/605) in 2008. The most prevalent mutation in each year was NRTI-associated resistance, with 11 (4.0%), 12 (4.0%), 21 (5.0%), 23 (5.2%), 28 (5.9%), and 23 (3.7%) cases, followed by PI- and NNRTI-associated mutations. PI-resistant major mutations were detected in 63 cases (2.5%), and NNRTI-associated mutations were detected only in 20 cases (0.8%). These data reflect the type of antiretrovirals being prescribed in treated population. In other words, NRTIs have a long history of being prescribed including the period of mono and dual therapy; thus, NRTIs have been more frequently used. As a consequence, NRTI-resistant HIV-1 has emerged and been transmitted more frequently to treatment-naïve patients. Regarding the drug-resistant mutations shown in Table 3, T215 revertants (T215X) (3.2%), M184I/V (0.5%), K103N (0.6%), and M46I/L (1.7%) accounted for the majority of detected mutations in contrast to other muta-

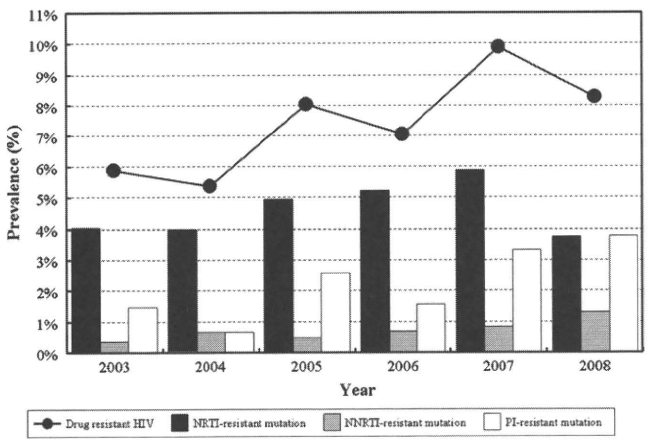

Fig. 1. Annual overall prevalence of drug-resistant HIV-1 (solid circles) in Japan increased in treatment-naïve patients in Japan from 2003 to 2008. The most prevalent mutation in each year was associated with resistance to nucleoside reverse transcriptase inhibitor (NRTI) treatment. Annual prevalence of drug-resistance mutations was categorized by antiretroviral drug class (NRTIs, solid black bars; non-nucleoside reverse transcriptase inhibitors [NNRTIs], horizontally striped bars; protease inhibitors [PIs], solid white bars). Drug-resistant HIV-1 was counted once even when the strain contained multiple drug-resistant mutations. Each drug-resistant mutation was counted even when multiple mutations were detected in one patient.

Table 3
Drug-resistant mutations in newly diagnosed HIV/AIDS patients, by class of antiretroviral drugs.

	6-Year total (2573)	
	n	(%)
NRTI[a]		
M41L	11	(0.4)
K65R	1	(0.0)
D67N/G/E	7	(0.3)
T69D	8	(0.3)
69INS	1	(0.0)
K70R/E	2	(0.1)
L74V/I	3	(0.1)
V75A/M	2	(0.1)
Y115F	3	(0.1)
M184V/I	12	(0.5)
L210W	5	(0.2)
T215X	81	(3.2)
K219Q/E/N/R	4	(0.2)
NNRTI[a]		
L100I	1	(0.0)
K101E	2	(0.1)
K103N	14	(0.6)
V106A/M	1	(0.0)
Y181C/I/V	3	(0.1)
P225H	1	(0.0)
P236L	1	(0.0)
PI[a]		
L24I	1	(0.0)
D30N	5	(0.2)
V32I	3	(0.1)
M46I/L	44	(1.7)
I47V/A	2	(0.1)
V82A/L	2	(0.1)
I85V	5	(0.2)
N88D/S	7	(0.3)
L90M	4	(0.2)

[a] Numbers of cases and the proportions in parentheses are listed.

Table 4
Predictive factors for transmission of drug-resistant HIV-1.

	Drug-resistant HIV-1 (*n*)		Odds ratio
	(+)	(−)	
Gender			
Male	183	2214	1.92
Female	7	163	
Nationality			
Japanese	173	2146	1.05
Non-Japanese	16	209	
Transmission category			
Male-to-male sexual contact	130	1643	0.91
High-risk heterosexual contact	37	484	
Sexual contact	15	60	
Other	1	40	
Unidentified[a]	11	152	
Subtype			
B	180	2014	2.36**
Non-B	11	291	
Unidentified	3	77	

[a] For calculation of odds ratio, unidentified cases were omitted.
** $p < 0.01$.

tions that were detected only sporadically throughout the study period (supplementary Table 2).

Analysis of possible predictive factors for transmission of drug-resistant HIV-1 showed that individuals infected with subtype B HIV-1 had a significantly higher tendency to harbor drug-resistant HIV-1 than non-B subtypes (OR = 2.36; 95% CI = 1.27–4.88; $p < 0.01$) (Table 4). Other possible predictive factors, including male gender (OR = 1.92; 95% CI = 0.89–4.93; $p = 0.1$), Japanese nationality (OR = 1.05; 95% CI = 0.62–1.92; $p = 1$), and MSM behavior (OR = 0.91; 95% CI 0.66–1.26; $p = 0.57$), were not significant predictive factors in our study population. These results indicate that the chance of getting infected with drug-resistant HIV-1 was the same for anyone regardless of gender, nationality, or risk behavior.

3.4. MSM are diagnosed earlier than heterosexually infected individuals

To examine awareness of HIV infection, especially of risk behavior, and to characterize HIV-testing patterns among the HIV-infected population, we estimated the time of seroconversion by quantifying the amount of anti-HIV antibody in plasma samples. Of 640 randomly selected samples in 2007 and 2008, 233 (36.4%) were classified by BED assay with a cut-off value of 0.8 as recently infected (<155-day seroconversion), while the remaining 407 (63.4%) were classified as not recently infected (Table 5). For the recently and not recently infected groups, the average $CD4^+$ T cell count and HIV-1 viral load were 285 and 215 cells/μL and 5.1×10^5 and 1.4×10^5 copies/mL, respectively. Recently infected individuals were shown by ANCOVA with $CD4^+$ T cell counts as the covariate, to have significantly higher HIV-1 viral loads than not recently infected cases (Fig. 2). These data support that the BED assay had precisely determined early infected cases.

With respect to risk behavior, the highest rate of recent infection was in MSM (39.2%), followed by either homo- or heterosexual contacts (38.9%), and heterosexual contacts (25.0%). No patients infected through a risk behavior other than sexual contacts were categorized as recently infected. Whereas 37.8% of male patients were determined to be recently infected, only 13.8% of female patients were categorized as recently infected. These findings were reinforced by statistical analysis. Recent HIV-1 infection was significantly predicted by male gender (OR = 3.79; 95% CI 1.29–15.17; $p < 0.01$), MSM behavior (OR = 1.67; 95% CI = 1.11–2.54; $p = 0.01$), Japanese nationality (OR = 2.31; 95% CI 1.20–4.76; $p < 0.01$), and infection with subtype B HIV-1 (OR = 5.64; 95% CI = 2.37–16.33; $p < 0.01$) (Table 5). In other words, Japanese males, especially those who were MSM, were more aware of being at high risk of HIV-1 infection and got tested more often than non-Japanese. In contrast, females, individuals of non-Japanese origin, heterosexuals, and non-subtype-B-infected persons, had low awareness of the risks of HIV-1 infection.

Table 5
Predictive factors for recent or not-recent seroconversion determined by BED assay, $n = 640$.

	Seroconversion (*n*)		Odds ratio
	Recent ($n = 233$)	Not recent ($n = 407$)	
Gender			
Male	229	377	3.79**
Female	4	25	
Unknown[b]	0	5	
Nationality			
Japanese	220	351	2.31**
Non-Japanese	13	48	
Unknown[b]	0	8	
Transmission category			
Male-to-male sexual contact	189	293	1.67[a,*]
High-risk heterosexual contact	24	70	
Sexual contact	7	11	
Other	0	4	
Unidentified[b]	13	29	
Subtype			
B	224	350	5.64**
Non-B	6	53	
Unidentified[b]	3	4	
Drug-resistant HIV			
Detected	14	37	0.64
Not detected	219	370	

[a] Odds ratio for the transmission category was calculated between male-to-male sexual contact and other categories which include high-risk heterosexual contact, sexual contact, and other.
[b] Unknown or unidentified cases were omitted in calculation of odds ratio.
* $p < 0.05$.
** $p < 0.01$.

Regarding associations between the time of diagnosis and drug-resistant HIV transmission event, time of diagnosis did not differ significantly between those harboring and those not harboring drug-resistant HIV-1 (OR = 0.64; 95% CI = 0.31–1.24; $p = 0.18$) (Table 5), suggesting that transmission of drug-resistant HIV-1 is not a recent trend, but has been ongoing since the first antiretroviral, AZT, was introduced in 1986.

4. Discussion

Our study results show that the proportion of drug-resistant HIV-1 among newly diagnosed cases in Japan increased slightly (by 2.4%) from 2003 to 2008, with fluctuations from year to year. Drug-resistant HIV-1 in HAART-naïve patients are transmitted from HAART-experienced patients with inadequate adherence or from other treatment-naïve individuals with drug-resistant strains, but not yet diagnosed or tested for drug-resistant HIV-1 (de Mendoza et al., 2005). Hence, drug-resistant mutations detected in the naïve population should be tightly related to trends in antiretroviral use in the treated population. Antiretrovirals available in the early days of the HAART era, especially, had short half-lives and low genetic barriers for drug resistance acquisition, making the viruses easily resistance prone. On the other hand, new antiretroviral drugs, such as lopinavir, atazanavir, amprenavir and darunavir, have been developed so that they have improved pharmacokinetics and higher genetic barriers, thus the viruses have less chance of developing drug resistance (Dunn et al., 2008; Lima et al., 2008; Zajdenverg et al., 2009). In the present study, we found that drug-resistant mutations detected among treatment-naïve patients were

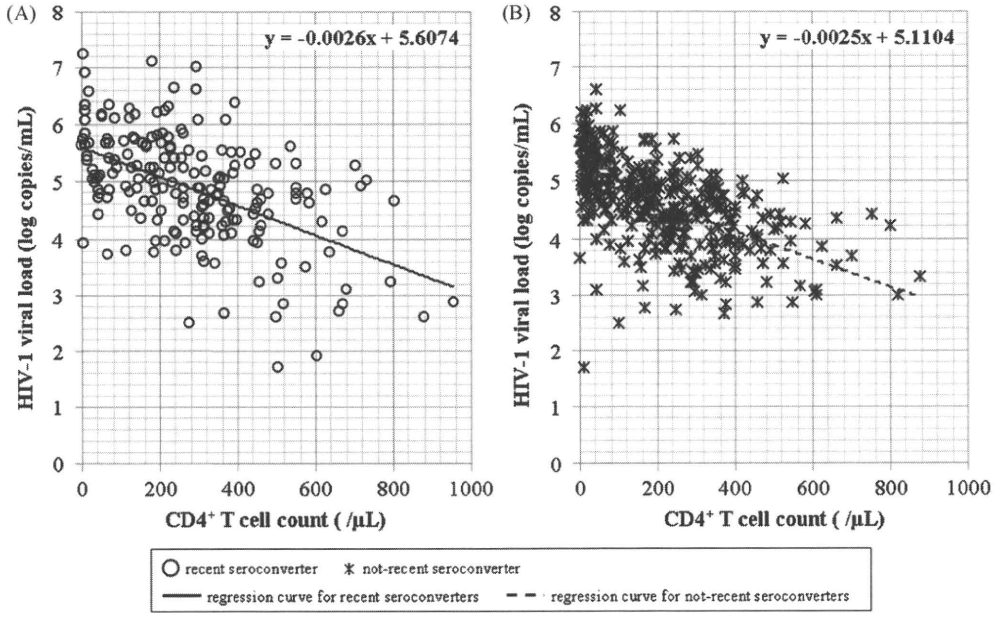

Fig. 2. Scatter plots of viral load and CD4+ T cell counts for (A) recently seroconverted patients (○), and (B) not recently seroconverted patients (*) determined by BED assay. Regression curves and their equations are shown for each group.

associated especially with antiretrovirals used prior to and early in the HAART era. It should be noted that contrary to the reports from the United States and many of European countries (Audelin et al., 2009; Vercauteren et al., 2009; Wheeler et al., 2010), the prevalence of NNRTI-resistant variants have been determined to be low in Japan, less than 1% in the study period 2003–2007 and 1.3% in 2008 being the highest. This difference is due to the situation in Japan that delavirdine had never been used and even nevirapine is only rarely prescribed. Nonetheless, strains with T215X, M46I/L, K103N, and M184V/I mutations were detected every year, suggesting that these strains are stably maintained in individuals and in high-risk populations even under antiretroviral drug-free environments. This finding is supported by the insignificant difference in prevalence of drug-resistant HIV-1 between recently and not recently infected groups. These results raise the concern that such drug-resistant strains may have become some epidemic strains actively transmitted among newly diagnosed HIV/AIDS patients. Furthermore, considering the presence of low frequent variants, the prevalence of drug-resistant mutations in this report may be higher if more sensitive techniques, such as allele-specific PCR and ultra-deep sequencing, are applied to test the samples (Halvas et al., 2010; Varghese et al., 2009). Further studies employing such techniques are needed to understand the detailed epidemic in Japan.

In investigating predictive factors for transmission of drug-resistant strains, we found that the only predictive factor was subtype B HIV-1 (OR = 2.36, $p < 0.01$). The lower transmission risk of drug-resistant strains in non-B HIV-1 can be explained by patients' countries of origin. We observed a significant relationship between non-B subtype HIV-1 and non-Japanese patients, most of whom were from developing countries with limited access to antiretrovirals. Thus, our finding agrees with reports of low prevalence drug-resistant HIV-1 transmission in developing countries (Bártolo et al., 2009; Ishizaki et al., 2009; Mintsa-Ndong et al., 2009; Ndembi et al., 2008; Nouhin et al., 2009; Pillay et al., 2008).

Interestingly, a high proportion of Japanese MSM was diagnosed as recently infected compared to patients of non-Japanese origin, and females determined by BED assay. This result may be due to successful prevention programs targeting the MSM community, so that they have become more aware of their risks of HIV-1 infection. On the other hand, many of non-Japanese patients are seen at hospitals long after HIV infection is established. In addition, women tend to be ignorant of the risks of HIV infection, thus they are often diagnosed upon a prenatal HIV screening test.

Although MSM was not a predictive factor for transmission, this group included 130 cases with drug-resistant HIV-1, the highest prevalence among all the transmission categories. Therefore, those who are involved in prevention programs should take one step further to remind the MSM community about drug-resistant HIV-1 and the limited choice of effective antiretrovirals. HIV-1 transmission has been reported to be prevented in models that assessed the effect of HIV-1 testing for wider populations and immediate initiation of antiretroviral therapy (Granich et al., 2009). Although this model seems very appealing, our results suggest the importance of not forgetting the emergence and transmission of drug-resistant HIV-1 and the limited selection of antiretroviral drugs. It is important to continue surveying newly diagnosed HIV/AIDS patients to keep track of trends in drug-resistant HIV-1 transmission, to reveal high-risk populations with low awareness of HIV infection, to propose effective programs to prevent transmission of drug-resistant HIV-1, and to develop antiretroviral drugs with improved pharmacokinetics/pharmacodynamics. All these efforts may bring us one step closer to eradicating HIV-1.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.antiviral.2010.07.008.

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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

Junko Tanuma [a,c,*], Atsuko Hachiya [a], Kyoko Ishigaki [a], Hiroyuki Gatanaga [a], Trinh Thi Minh Lien [b], Nguyen Duc Hien [b], Nguyen Van Kinh [b], Mitsuo Kaku [c], Shinichi Oka [a]

[a] *AIDS Clinical Center, National Center for Global Health and Medicine, 1-21-1 Toyama, Shinjuku-ku, Tokyo 162-8655, Japan*
[b] *National Hospital for Tropical Diseases., 78 Giai Phong Street, Dong Da District, Hanoi, Vietnam*
[c] *Department of Infection Control and Laboratory Diagnostics, Internal Medicine, Tohoku University Graduate School of Medicine. 1-1 Seiryo-cho, Aoba-ku, Sendai, Japan*

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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

* Corresponding author. AIDS Clinical Center, National Center for Global Health and Medicine, 1-21-1 Toyama, Shinjuku, Tokyo 162-8655, Japan. Tel.: +81 3 3202 7181x5642; fax: +81 3 3202 7198.
E-mail address: jtanuma@acc.ncgm.go.jp (J. Tanuma).

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