

infected Japanese individuals who could be monitored from the early phase to the chronic phase of an HIV-1 infection, as well as the abilities of cross-reactive, 3R mutant-specific, and WT-specific CTLs to kill WT or 3R virus-infected cells and to suppress the replication of the WT or 3R virus. In addition, we investigated the accumulation of the 3R mutation in HIV-1-infected nonhemophiliac Japanese individuals, as well as in Japanese hemophiliacs who had been infected around 1983. The results clarified the role of CTLs recognizing the WT and/or 3R epitope in high accumulation of the 3R mutant in HIV-1-infected Japanese individuals.

MATERIALS AND METHODS

Samples from HIV-1-infected individuals. This study was approved by the ethics committee of Kumamoto University and the National Center for Global Health and Medicine. Informed consent was obtained from all individuals according to the Declaration of Helsinki. For sequence analysis, blood specimens were collected in EDTA. Plasma and peripheral blood mononuclear cells (PBMCs) were separated from whole blood. HLA types were determined by standard sequence-based genotyping. Twelve HLA-A*24:02⁺ individuals who could be monitored from the early to the chronic phase of an HIV-1 infection were recruited for CTL analysis. Early HIV-1 infection was confirmed by seroconversion within 6 months or by an increasing number and density of bands on Western blots. Four-hundred fifty-one chronically HIV-1-infected individuals were also recruited for sequence analysis.

Cells. C1R cells expressing HLA-A*24:02 (C1R-A2402) and 721.221 cells expressing CD4 and HLA-A*24:02 (721.221-CD4-A2402) were previously generated (27, 30). These cells were cultured in RPMI 1640 medium containing 5 to 10% fetal bovine serum (FBS) and 0.15 mg/ml hygromycin B. MAGIC-5 cells (CCR5-transfected HeLa-CD4/long terminal repeat- β -galactosidase [LTR- β -Gal] cells) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS as described previously (17).

Induction of Gag28-specific T cells. PBMCs from HIV-1-infected HLA-A*24:02⁺ individuals were stimulated with WT or 3R peptide (1 μ M) in culture medium (RPMI 1640 containing 10% FBS and 200 U/ml human recombinant interleukin-2 [rIL-2]). After 14 days, the cultured PBMCs were tested for gamma interferon (IFN- γ) production by performing an intracellular cytokine staining (ICC) assay.

ICC assay. C1R-A2402 cells were prepulsed or not with the WT or 3R peptide at concentrations from 0.1 to 1,000 nM at 37°C for 1 h and then were washed twice with RPMI 1640 containing 10% FBS. PBMCs cultured for 2 weeks after peptide stimulation were incubated with the C1R-A2402 cells in a 96-U plate (Nunc) at 37°C. Brefeldin A (10 μ g/ml) was added after a 2-h incubation, and then the cells were incubated for an additional 4 h. Subsequently, the cells were stained with Pacific-blue-conjugated anti-CD8 monoclonal antibody (MAb) (BD Biosciences) and 7-aminocoumarin D (7-AAD) (BD Biosciences) at 4°C for 30 min, after which the cells were fixed with 4% paraformaldehyde solution and rendered permeable with permeabilization buffer (0.1% saponin and 10% FBS in phosphate-buffered saline) at 4°C for 10 min. Thereafter the cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-IFN- γ MAb (BD Biosciences) at 4°C for 30 min and then washed twice with the permeabilization buffer. The percentage of CD8⁺ cells producing IFN- γ was analyzed by flow cytometry (FACSCanto II).

Generation of Gag28-specific CTL clones. Gag28-specific CTL clones were generated from Gag28-specific bulk-cultured T cells by limiting dilution in 96-U plates, together with 200 μ l of cloning mixture (1×10^6 irradiated allogeneic PBMCs from healthy donors and 1×10^5 irradiated C1R-A2402 cells prepulsed with the WT or 3R peptide at a concentration of 1 μ M in RPMI 1640 containing 10% FBS, 200 U/ml rIL-2, and 2.5% phytohemagglutinin [PHA] soup). After 14 to 21 days in culture, the growing cells were tested for cytotoxic activity by performing the standard

chromium release assay. Since TCRs on these CTL clones were not sequenced, it is still possible that they were oligonucleotide clones.

HIV-1 clones. An infectious provirus, HIV-1 pNL-432, was reported previously (1). NL-432gagSF2 and NL-432gagSF2-3R were previously generated (30).

Assay of cytotoxicity of CTL clones toward target cells prepulsed with the epitope peptide. The cytotoxic activities of Gag28-specific CTL clones were determined by use of the standard chromium release assay, as described previously (15). Briefly, 721.221-CD4-A2402 cells were incubated with 100 μ Ci of Na₂⁵¹CrO₄ in saline for 1 h and then washed 3 times with RPMI 1640 containing 10% newborn calf serum. The labeled target cells (2×10^3 /well) were prepulsed with the WT or 3R peptide at concentrations of 1 to 1,000 nM for 1 h and then cocultured at 37°C for 4 h with effector cells at an effector-to-target (E:T) ratio of 1:1 in 96-U plates (Nunc). The supernatants were collected and analyzed with a gamma counter. Spontaneous ⁵¹Cr release was determined by measuring the counts per minute in supernatants from wells containing only target cells (cpm spn). Maximum ⁵¹Cr release was determined by measuring the cpm in supernatants from wells containing target cells in the presence of 2.5% Triton X-100 (cpm max). Specific lysis was defined as $(\text{cpm exp} - \text{cpm spn}) / (\text{cpm max} - \text{cpm spn}) \times 100$, where "cpm exp" is the counts per minute in the supernatant in the wells containing both target and effector cells.

Assay of cytotoxicity of CTL clones toward target cells infected with HIV-1. 721.221-CD4-A2402 cells were infected with WT or 3R virus, and then the infection rates were determined by detecting intracellular p24 antigen (Ag)-positive cells stained with FITC-conjugated anti-p24 Ag MAb (KC57-FITC; BD Biosciences). When approximately 50% of the total cells were p24 Ag-positive cells, they were used as target cells. The ⁵¹Cr-labeled target cells (2×10^3 /well) were cocultured with effector cells at E:T ratios of 0:1 to 2:1 in 96-U plates at 37°C for 6 h. The supernatants were collected and analyzed with a gamma counter.

Generation of HLA-peptide tetrameric complexes. HLA class I-peptide tetrameric complexes (tetramers) were synthesized as previously described (3). The WT or 3R peptide was added to the refolding solution containing the biotinylation sequence-tagged extracellular domain of the HLA-A*24:02 molecule and β 2 microglobulin. The purified monomer complexes were mixed with phycoerythrin (PE)-labeled streptavidin (Molecular Probes) at a molar ratio of 4:1.

Tetramer binding assay. CTL clones were stained with PE-conjugated tetramer at concentrations of 1 to 100 nM at 37°C for 30 min. After 2 washes with RPMI 1640 containing 10% FBS (R10), the cells were stained with FITC-conjugated anti-CD8 MAb and 7-AAD at 4°C for 30 min. Thereafter, the cells were washed twice with R10 and then analyzed by flow cytometry (FACSCanto II). The mean fluorescence intensity (MFI) of tetramer-positive cells among CD8-positive cells was calculated.

Replication suppression assay. The ability of Gag28-specific CTLs to suppress HIV-1 replication was examined as previously described (43). CD4⁺ T cells were isolated from PBMCs of healthy HLA-A*24:02⁺ donors and incubated with a given HIV-1 clone at 37°C for 6 h. After 3 washes with R10, the cells (3×10^4 /well) were cocultured with Gag28-specific CTL clones at E:T ratios of 0.1:1 to 1:1 in R10 containing 1% nonessential amino acid solution and, 1% 100 mM sodium pyruvate (complete medium) plus 200 U/ml rIL-2. From day 3 to day 7 postinfection, a 30- μ l volume of culture supernatant was collected, and the volume removed was replaced with fresh medium. The concentration of p24 Ag was measured by using an enzyme-linked immunosorbent assay (ELISA) (HIV-1-p24-Ag ELISA kit; ZeptoMetrix).

Replication kinetics assay. The replication kinetics of the WT and 3R viruses were examined as previously described (17). After CD4⁺ T cells (2×10^6) had been exposed to each infectious virus preparation (500 blue cell-forming units in MAGIC-5 cells) for 2 h and washed twice with R10, they were cultured in 1 ml of R10 containing 1% nonessential amino acid solution and 1% 100 mM sodium pyruvate (complete medium) plus 200 U/ml rIL-2. Then, 0.1 ml of the culture supernatant was collected from

day 2 to day 10 postinfection, and the volume removed was replaced with fresh medium. The concentration of p24 Ag in the supernatant was measured by using ELISA. Replication kinetics assays were performed in triplicate.

Sequence of autologous virus. Viral RNA was extracted from plasma samples from HIV-1-infected individuals by using a QIAamp MinElute virus spin kit (Qiagen). For clone sequencing, cDNA was synthesized from the RNA with SuperScript III and Random Primers (Invitrogen), and the Gag region was amplified by nested PCR with *Taq* DNA polymerase (Promega). Then, the PCR products were gel purified and cloned with a TOPO TA cloning kit (Invitrogen). For bulk sequencing, the Gag region was amplified from the RNA by using the SuperScript III One-Step RT-PCR System with Platinum *Taq* DNA Polymerase (Invitrogen) and Gag-specific primers, and then the second PCR was done. We prepared the Gag-specific primer sets shown below. For clone sequencing, 5'-TTTTT GACTAGCGGAGGCTAGAA-3' and 5'-CACAAATAGAGGGTTGCTAC TGT-3' were used for the first PCR and 5'-GGGTGCGAGAGCGTCCG TATTAAGC-3' and 5'-TAAGTTCTTCTGATCCTGTCTG-3' for the second PCR. For bulk sequence, 5'-TCTCTCGACGCAGGACTC-3' and 5'-AGGGTTCCTTTGGTCCTTGT-3' were employed for the reverse transcription (RT)-PCR and 5'-TCTCTCGACGCAGGACTC-3' and 5'-TCTCCTACTGGGATAGGTG-3' for the second PCR. All DNA sequencing was performed by using a BigDye Terminator cycle-sequencing kit (Applied Biosystems) and an ABI Prism 310 or 3100 genetic analyzer.

RESULTS

Selection of the 3R mutation by WT epitope-specific CD8⁺ T cells in individuals infected with WT virus. We investigated 12 HIV-1-infected HLA-A*24:02⁺ individuals who could be monitored from the early to the chronic phases of their infections. We first analyzed the sequence of the Gag28 epitope at an early phase in the 12 HIV-1-infected HLA-A*24:02⁺ individuals. The WT sequence of the Gag28 epitope was detected in 4 of these individuals, whereas 3R was found in the other 8, suggesting that the former and the latter individuals had been infected with WT and 3R viruses, respectively (Table 1). This is consistent with a previous finding that the 3R mutant is found in approximately 70% of HIV-1-infected HLA-A*24:02⁺ individuals (30). We investigated the elicitation of Gag28-specific CD8⁺ T cells in the individuals infected with WT virus. PBMCs from these individuals at early and chronic phases were stimulated with WT or 3R peptide and then cultured for 2 weeks. The frequency of Gag28-specific CD8⁺ T cells among the cultured cells was measured by performing the ICC assay using WT and 3R peptides. Gag28-specific CD8⁺ T cells were detected at the early phase in 3 of the 4 individuals when the PBMCs were stimulated with WT peptide (Table 2). In 2 individuals, i.e., KI-092 and KI-161, Gag28-specific CD8⁺ T cells were much more WT specific than 3R mutant specific, whereas in KI-158 they recognized both peptides, but especially the WT peptide (Fig. 1). On the other hand, cross-reactive CD8⁺ T cells were induced in KI-092 and KI-161 when their PBMCs had been stimulated with 3R peptide, although the frequency of cross-reactive CD8⁺ T cells induced by stimulation with 3R peptide was lower than that of WT-specific cells induced by stimulation with WT peptide. The 3R peptide failed to induce Gag28-specific CD8⁺ T cells in PBMCs from KI-158. Thus, WT-specific CD8⁺ T cells were predominantly elicited at an early phase in the individuals infected with WT virus, although a small but significant number of cross-reactive T cells were also elicited in them.

To clarify the specificity of Gag28-specific CD8⁺ T cells at the early phase in KI-092 and KI-161, we generated Gag28-specific CTL clones by stimulating early-phase PBMCs from KI-092 and

TABLE 1 Sequence at Gag30 in 12 HLA-A*24:02⁺ individuals with an early-phase HIV-1 infection

Patient ID ^a	Sampling date (mo/day/yr)	Gag30 sequence	Method	
KI-091	12/13/2000	3R	Cloning	
	12/27/2000	3R	Direct	
	1/7/2002	3R	Direct	
	7/9/2003	3R	Cloning	
	9/29/2004	3R	Cloning	
KI-092	8/4/2005	3R	Cloning	
	1/22/2001	WT	Cloning	
	11/21/2001	WT	Cloning	
	12/10/2002	WT/3R	Cloning	
KI-102	8/14/2003	3R	Cloning	
	5/11/2001	WT	Direct	
	7/5/2004	WT	Direct	
KI-126	3/28/2005	WT	Direct	
	7/19/2001	3R	Direct	
	1/18/2002	3R	Direct	
	11/15/2004	3R	Direct	
KI-134	9/12/2005	3R	Direct	
	10/25/2001	3R	Direct	
KI-136	6/30/2004	3R	Direct	
	10/29/2001	3R	Direct	
	7/10/2003	3R	Direct	
KI-140	11/08/2001	3R	Direct	
KI-151	5/2/2001	3R	Direct	
	8/28/2003	3R	Direct	
KI-154	4/12/2002	3R	Direct	
KI-158	6/14/2002	WT	Direct	
	10/11/2002	WT	Direct	
	8/25/2003	WT	Direct	
	11/14/2003	WT/3R	Direct	
	2/23/2004	3R/WT	Direct	
	11/1/2004	3R	Direct	
	4/4/2005	3R	Direct	
	KI-161	2/15/2002	WT	Direct
		9/12/2002	WT	Direct
		3/4/2003	WT	Direct
9/30/2003		WT/3R	Direct	
KI-163	5/6/2004	3R	Direct	
	1/27/2005	3R	Direct	
	6/16/2005	3R	Cloning	
KI-163	8/30/2002	3R	Direct	
	9/27/2004	3R	Direct	

^a ID, identifier.

KI-161 with the WT peptide. The CTL clones from KI-092 showed a much greater ability to kill cells prepulsed with WT peptide than to kill those prepulsed with the 3R peptide (Fig. 2A), suggesting that they were WT-specific CTLs. To further clarify the specificity of these T cell clones, we investigated the binding affinity of the clones for WT peptide-binding HLA-A*24:02 tetramer (WT tetramer) and 3R peptide-binding HLA-A*24:02 tetramer (3R tetramer). These clones exhibited much greater binding ability to the WT tetramer than to the 3R tetramer (Fig. 2B). These results together indicate that these were WT-specific CTL clones. We further analyzed the abilities of these clones to recognize HIV-1-infected cells. These CTL clones effectively killed WT-virus-infected cells, but not the 3R virus-infected cells (Fig. 2C), and showed the ability to suppress the replication of WT virus, but not to suppress that of the 3R virus (Fig. 2D). WT-specific CD8⁺ T cell

TABLE 2 Responses of CD8⁺ T cells from individuals infected with WT virus to WT or 3R peptide

Patient ID	Virus sequence [mo/day/yr (type)]		PBMC sampling date (mo/day/yr)	PBMCs cultured with:	% IFN- γ -producing cells specific for each peptide among CD8 ⁺ T cells ^a		
	Early phase	Chronic phase			Without	WT	3R
KI-092	1/22/2001 (WT)	8/14/2003 (3R)	5/24/2001	WT	0.2	34.4	13.7
			2/3/2003	3R	0.1	12.1	16.8
				WT	0.2	5.8	4.2
KI-102	5/11/2001 (WT)	3/28/2005 (WT)	7/11/2001	3R	0.6	0.3	0.3
			7/5/2004	WT	1.0	0.6	1.1
				3R	1.1	1.5	2.0
KI-158	6/14/2002 (WT)	4/4/2005 (3R)	10/11/2002	WT	0.2	28.7	9.3
			4/4/2005	3R	0.6	0.7	0.6
				WT	1.4	19.3	24.6
KI-161	2/15/2002 (WT)	6/16/2005 (3R)	7/26/2002	3R	0.1	0.5	0.4
			5/6/2004	WT	0.3	23.3	23.8
				3R	0.4	18.8	20.9
				WT	0.0	74.5	8.0
				3R	0.2	55.1	41.8
				WT	0.1	21.4	4.9
				3R	0.2	42.5	43.9

^a Without, without peptide. Boldface, positive IFN- γ -producing response.

clones established from early-phase PBMCs of KI-161 also showed a similar ability to kill WT virus-infected and 3R virus-infected cells (Fig. 3). In these individuals, the 3R mutant virus became dominant 1 to 2 years after the early phase (Table 1). Taken together, these findings suggest that the 3R mutation was selected by WT-specific CTLs.

The 3R virus was not detected by approximately 4 years postinfection in KI-102, who had been infected with the WT virus (Table 1). This individual did not have Gag28-specific CD8⁺ T cells at an early phase of the HIV-1 infection (Fig. 1). Interestingly, only WT-specific CD8⁺ T cells were induced from PBMCs of this patient 2.5 year later. Thus, WT-specific CD8⁺ T cells did not select 3R within about 2 years after the WT-specific CD8⁺ T cells had been elicited in the patient.

Cross-reactive CD8⁺ T cells in individuals who had been infected with WT virus and had selected 3R virus. We investigated whether the 3R-specific or cross-reactive CD8⁺ T cells were elicited after the 3R mutant had been selected in individuals who had been infected with the WT virus. In KI-158, no Gag28-specific CD8⁺ T cells were induced from early-phase PBMCs stimulated with the 3R peptide, whereas cross-reactive CD8⁺ T cells were induced from chronic-phase PBMCs stimulated with WT peptide or 3R peptide (Fig. 1). In KI-161, Gag28-specific CD8⁺ T cells recognizing WT peptide more than the 3R peptide were induced from early-phase PBMCs stimulated with WT peptide or the 3R peptide, whereas cross-reactive CD8⁺ T cells were predominantly induced from chronic-phase PBMCs stimulated with the 3R peptide (Fig. 1). These results indicate that cross-reactive CD8⁺ T cells became dominant in the Gag28-specific CD8⁺ T cell population after the emergence of the 3R virus in these 2 individuals.

To investigate the function of these cross-reactive CD8⁺ T cells, we generated Gag28-specific CTL clones from PBMCs at a chronic phase in KI-161 by stimulating them with the 3R peptide. The CTL clones evenly recognized both WT and the 3R peptides (Fig. 3A) and showed the same binding affinity to the 2 tetramers (Fig. 3B). These results suggest that the two peptides had the same

binding affinity for HLA-A*24:02. They effectively killed WT-virus-infected cells and weakly killed the 3R virus-infected cells (Fig. 3C), whereas they suppressed the replication of the WT virus but not that of the 3R virus (Fig. 3D). These results indicate that these cross-reactive CTLs contributed to the selection of the 3R virus. In addition, the results strongly suggest weak presentation of the 3R peptide in the cells infected with 3R virus, because the cross-reactive CTL clones had TCR with the same binding affinity for both HLA-A*24:02-WT peptide and HLA-A*24:02-3R peptide complexes and because WT and 3R peptides had the same binding affinity for HLA-A*24:02. This reduced presentation may have affected the control of 3R virus by the cross-reactive CTLs.

Gag28-specific T cell repertoire in an individual infected with WT virus. The results in Fig. 1 suggest that both WT-specific and cross-reactive CD8⁺ T cells were elicited at an early phase of HIV-1 infection in 3 individuals infected with WT virus (KI-092, KI-158, and KI-161). To characterize Gag28-specific CTLs elicited at that time, we established Gag28-specific CTL clones from PBMCs at an early phase in KI-161 by stimulating them with the WT peptide. We found 3 types of CTL clones among the 8 clones analyzed. As shown in Fig. 3A, 3 clones effectively recognized the WT peptide but not the 3R peptide (WT specific), 3 clones recognized the WT peptide more than the 3R peptide (WT dominant), and 2 clones evenly recognized both peptides (cross-reactive). We next investigated the binding affinity of TCRs on these clones to WT tetramer and 3R tetramer. The results confirmed the specificity of these 3 types of CTL clones (Fig. 3B). These results together indicate that KI-161 had a multiple T cell repertoire for the Gag28 epitope before the 3R virus had been selected.

Next, we analyzed the abilities of these T cell clones to kill HIV-1-infected cells. The WT-specific and WT-dominant CTL clones effectively killed the target cells infected with WT virus but failed to kill those infected with the 3R virus (Fig. 3C, left and right graphs under early phase). On the other hand, cross-reactive CTL clones weakly killed the target cells infected with the 3R virus and effectively killed those infected with the WT virus (Fig. 3C, middle

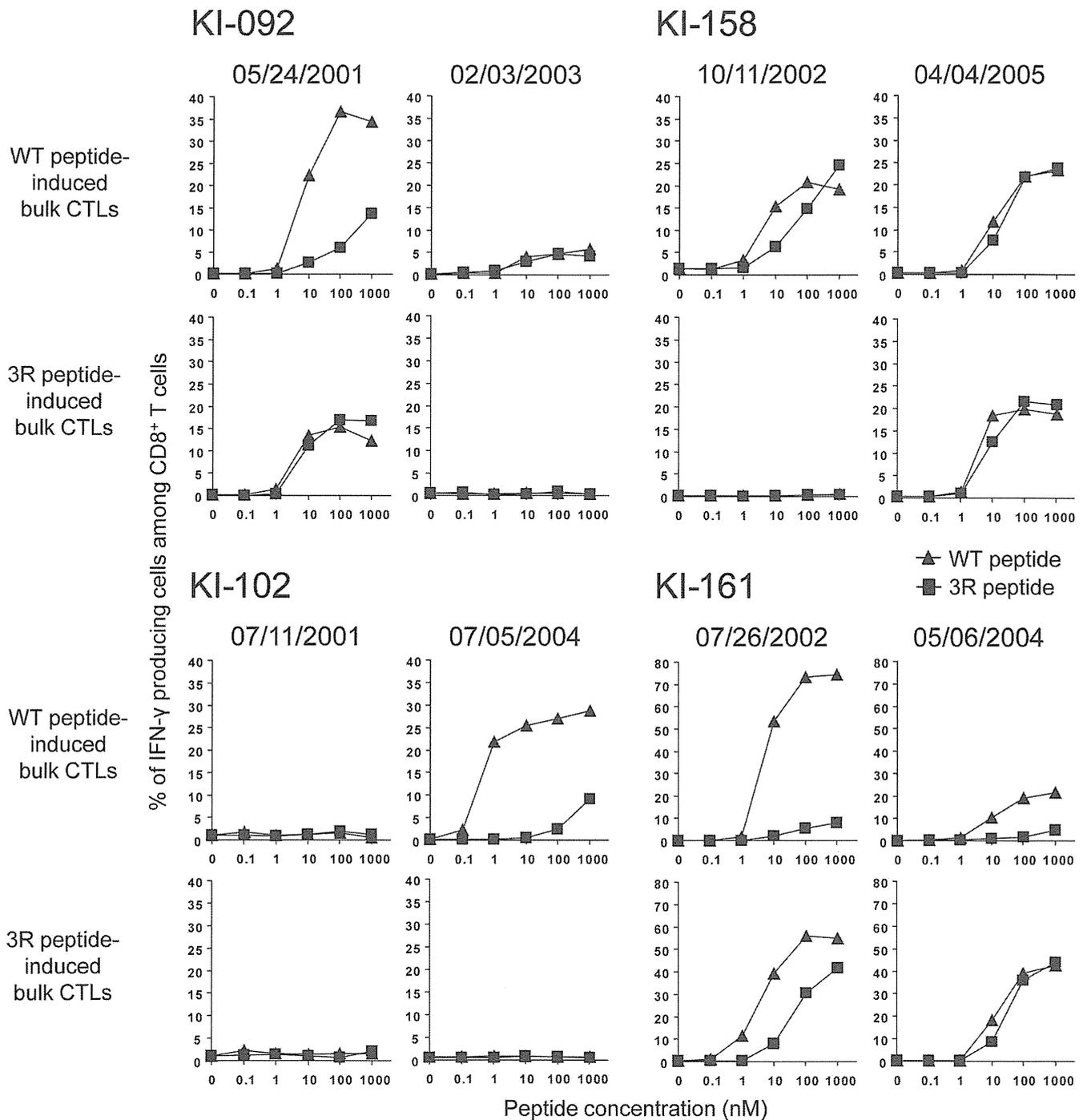


FIG 1 Gag28-specific CD8⁺ T cells from individuals infected with WT virus at early and chronic phases. Gag28-specific CD8⁺ T cells were induced by stimulating PBMCs from early and chronic phases in 4 WT-virus-infected HLA-A*24:02⁺ individuals with WT or 3R peptide. The responses of these bulk-cultured cells to C1R-A2402 cells prepulsed with WT or 3R peptide at concentrations of 0.1 to 1,000 nM were analyzed by using the ICC assay.

graphs under early phase). Then, we analyzed the abilities of these CTL clones to suppress HIV-1 replication. Both WT-specific and cross-reactive CTL clones effectively suppressed the replication of the WT virus, whereas WT-specific and cross-reactive CTL clones exhibited no and weak ability, respectively, to suppress that of the 3R virus (Fig. 3D). These results indicate that WT-specific and cross-reactive CTLs could suppress the replication of the WT virus

but that the former CTLs could not suppress the 3R virus *in vivo*. The latter CTLs may weakly suppress 3R virus *in vivo*. Interestingly, the WT-dominant CTL clones exhibited much weaker ability to suppress the replication of WT virus than did the WT-specific and cross-reactive CTLs (Fig. 3D), although no difference in killing activity against WT-virus-infected cells was found among these 3 CTL clones. Overall, KI-161 had a multiple Gag28-

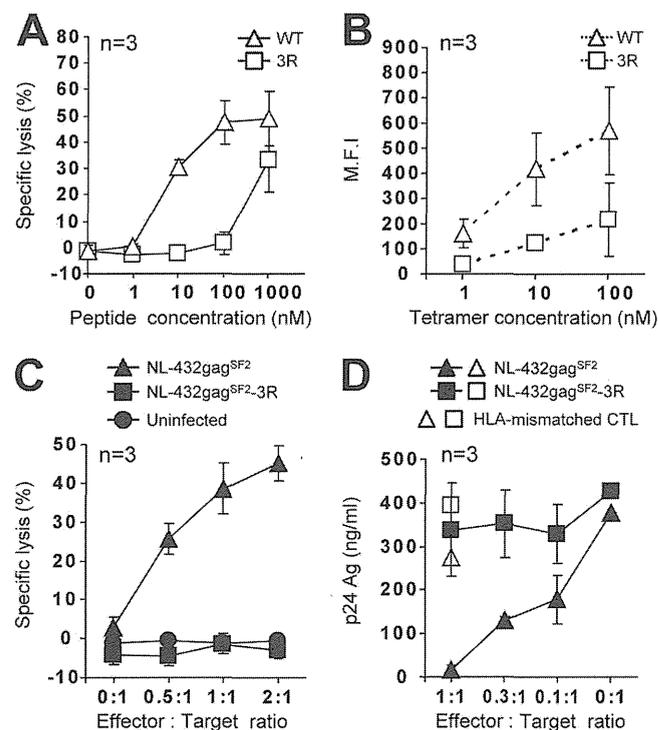


FIG 2 Antiviral activity of Gag28-specific CTL clones generated from early-phase PBMCs from patient KI-092, infected with WT virus. Gag28-specific CTL clones were generated from early-phase PBMCs from KI-092 by stimulating them with WT peptide. The activities of 3 CTL clones ($n = 3$) were analyzed. (A) Cytotoxic activity toward 721.221-CD4-A2402 cells prepulsed with the WT or 3R peptide at concentrations of 1 to 1,000 nM. The cytotoxic activity was measured at an E:T ratio of 1:1. (B) Binding affinity to WT and 3R tetramers at concentrations of 1 to 100 nM. The MFI values of the T cell clones are shown. (C) Cytotoxic activity against 721.221-CD4-A2402 cells infected with NL-432gag^{SF2} (WT virus) or NL-432gag^{SF2}-3R (3R virus). WT-virus-infected (49.1% of total cells were p24 Ag⁺) and 3R virus-infected (48.6% of total cells were p24 Ag⁺) cells were used as target cells. The cytotoxic activity was measured at E:T ratios of 0.5:1, 1:1, and 2:1. (D) Abilities of the clones to suppress the replication of WT or 3R viruses. The ability was tested at different E:T ratios. The error bars indicate standard deviations.

specific CTL repertoire at an early phase of HIV-1 infection, but only 2 types of Gag28-specific CTLs, which were the majority among the Gag28-specific CTLs, contributed to the suppression of WT virus replication.

Cross-reactive CD8⁺ T cells and 3R-specific CD8⁺ T cells in individuals who were infected with 3R virus. Next, we analyzed the elicitation of Gag28-specific CD8⁺ T cells in 5 individuals infected with the 3R virus. Gag28-specific CD8⁺ T cells were detected at both early and chronic phases in 3 individuals, whereas they were found at only the chronic phase in the other 2 (Table 3). Cross-reactive CD8⁺ T cells were induced by stimulating KI-091 PBMCs from both early and chronic phases, not only with 3R peptide, but also with WT peptide. To characterize Gag28-specific CD8⁺ T cells in KI-091, we generated Gag28-specific CTL clones from PBMCs at a chronic phase in KI-091 by stimulating them with 3R peptide. We investigated the recognition of 3 CTL clones for WT and 3R peptides. These CTL clones evenly recognized both peptides (Fig. 4A) and revealed the same binding affinity for the 2 tetramers (Fig. 4B), indicating that they were cross-reactive CTLs. They moderately killed target cells infected with either WT or 3R

virus (Fig. 4C) but did not suppress the replication of the WT and 3R viruses (Fig. 4D). Thus, Gag28-specific CD8⁺ T cells elicited in KI-091 had no ability to suppress the replication of WT and 3R viruses. Further analysis of 13 other clones revealed similar characteristics (data not shown), supporting the data indicating that cross-reactive CTLs were predominantly elicited in KI-091.

In the chronic phase, KI-091 had cross-reactive CD8⁺ T cells, whereas 3R-specific CD8⁺ T cells were found in 4 other individuals (Table 3). To characterize these 3R-specific CD8⁺ T cells, we generated 3R-specific CTL clones from KI-163 PBMCs at the chronic phase by stimulating them with 3R peptide. All 3 clones recognized the 3R peptide much more effectively than the WT peptide (Fig. 4A). These CTL clones bound to 3R tetramer, but not to WT tetramer (Fig. 4B), indicating that these CTL clones carried a 3R-specific TCR. In addition, we analyzed the abilities of these CTL clones to recognize virus-infected cells and found that they effectively killed target cells infected with 3R virus, but not those infected with WT virus (Fig. 4C). However, they failed to suppress the replication of either 3R or WT virus (Fig. 4D). These results indicate that Gag28-specific CD8⁺ T cells elicited in all individuals infected with 3R virus had no ability to suppress the replication of WT or 3R virus. Thus, Gag28-specific CD8⁺ T cells seem to have failed to control the 3R virus, although they were elicited in individuals infected with the 3R virus.

High accumulation of the 3R variant in the Japanese population. The results described above strongly suggest that WT-specific and cross-reactive CD8⁺ T cells selected the 3R mutation in the individuals infected with the WT virus and that 3R-specific and cross-reactive CD8⁺ T cells failed to control the 3R virus in the individuals infected with it. Therefore, we assume that this 3R mutation has accumulated in the HLA-A*24:02⁺ individuals. In addition, since HLA-A*24:02 is found in approximately 70% of Japanese, we speculate that the mutation has accumulated to high levels in the Japanese population.

A previous study analyzed the frequency of 3R in only 32 HLA-A*24:02⁺ and 26 HLA-A*24:02⁻ individuals chronically infected with HIV-1 and showed that the frequency of 3R was significantly higher in HLA-A*24:02⁺ individuals than in the HLA-A*24:02⁻ individuals (30). To confirm the association of this mutation with HLA-A*24:02, we analyzed a large number of chronically HIV-1-infected nonhemophiliac individuals (220 HLA-A*24:02⁺ and 154 HLA-A*24:02⁻ individuals) recruited from April 2008 to March 2011 (2008 to 2011 cohort). The results confirmed that the frequency of 3R was significantly higher in HLA-A*24:02⁺ individuals than in the HLA-A*24:02⁻ individuals ($P < 0.0005$) (Fig. 5). Since 3R was found in 74.7% of the HLA-A*24:02⁻ individuals in this cohort, we speculate that the mutation has been accumulating in the Japanese population. Therefore, we analyzed HIV-1-infected nonhemophiliac Japanese individuals who had been recruited from 1996 to 2002 (1996 to 2002 cohort), as well as Japanese hemophiliacs who had been infected around 1983 (hemophiliac cohort), and then compared them to the 2008 to 2011 cohort (Fig. 5). The association of this mutation with HLA-A*24:02 was also found in both the 1996 to 2002 cohort and the hemophiliac cohort ($P < 0.01$ and $P = 7.4 \times 10^{-7}$, respectively). The frequency of this mutation in HLA-A*24:02⁻ individuals significantly increased from 0% in the hemophiliac cohort to 50.0% in the 1996 to 2002 cohort ($P = 0.0084$) and to 74.7% in the 2008 to 2011 cohort ($P = 2.6 \times 10^{-7}$). These results indicate that the 3R mutation was strongly selected by Gag28-specific CTLs and has

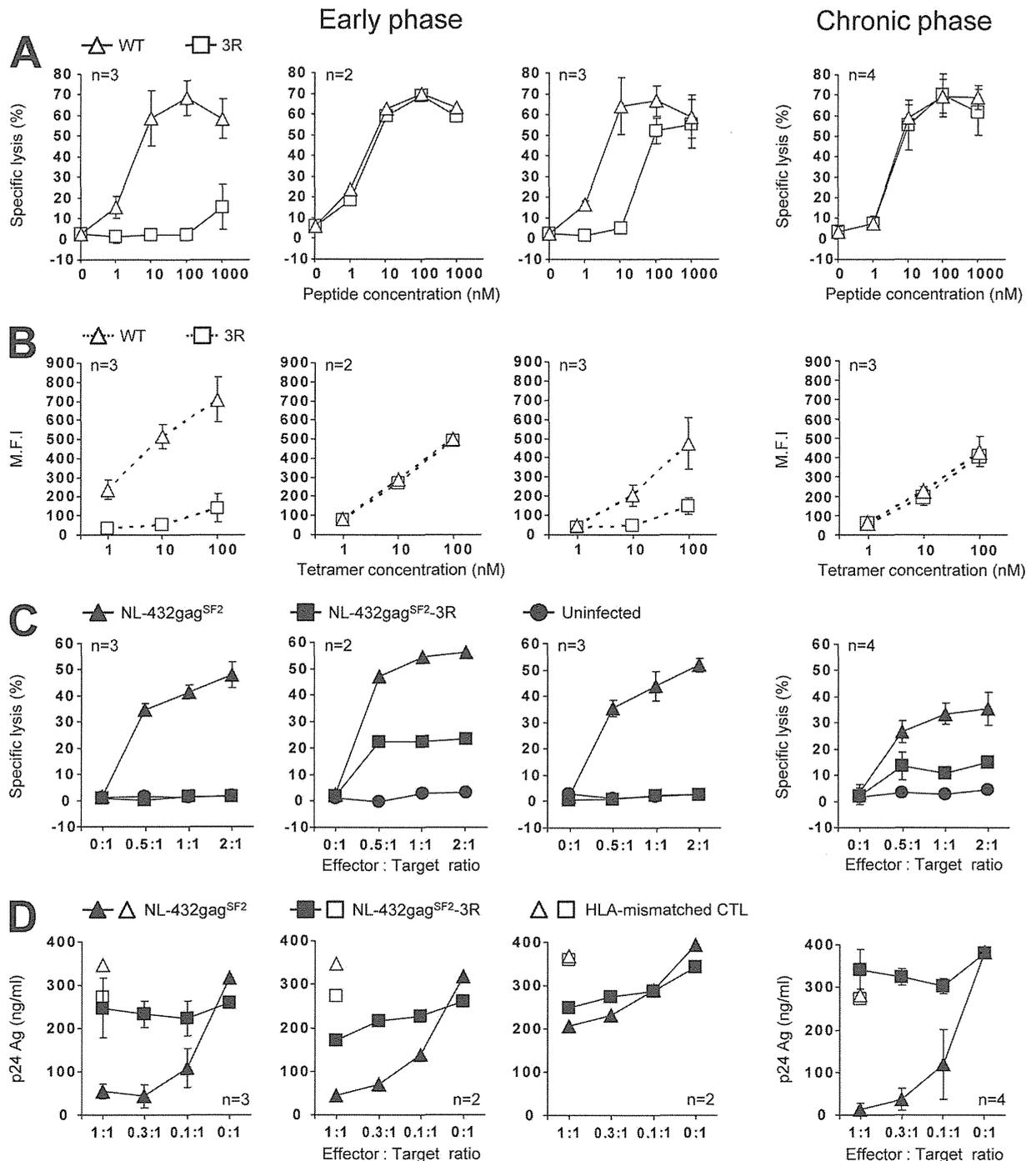


FIG 3 Antiviral activities of Gag28-specific CTL clones generated from PBMCs of patient KI-161, infected with WT virus. Gag28-specific CTL clones were generated from early-phase and chronic-phase PBMCs isolated from KI-161 after stimulating them with the WT and 3R peptides, respectively. Three types of Gag28-36-specific CTL clones, i.e., WT specific (left), cross-reactive (middle), and WT dominant (right), were generated from the early-phase PBMCs. (A) Cytotoxic activity against 721.221-CD4-A2402 cells prepulsed with the WT or 3R peptide at concentrations of 1 to 1,000 nM. The cytotoxic activity was measured at an E:T ratio of 1:1. (B) Binding affinity toward WT and 3R tetramers at concentrations of 1 to 100 nM. The MFIs of the T cell clones are shown. (C) Cytotoxic activity against 721.221-CD4-A2402 cells infected with WT virus or 3R virus. WT-virus-infected (49.0% of total cells were p24 Ag⁺) and 3R-virus-infected (50.0% of total cells were p24 Ag⁺) cells were used as target cells. The cytotoxic activity was measured at E:T ratios of 0.5:1, 1:1, and 2:1. (D) Abilities of the clones to suppress the replication of WT or 3R virus. The ability was tested at different E:T ratios. n, number of clones tested. The error bars indicate standard deviations.

been accumulating during the past 30 years in the Japanese population.

It is well known that some escape mutations affect replication capacity and that HIV-1 containing such mutations reverts to WT

in individuals not carrying HLA class I restriction alleles (23, 32). We previously showed that the 3R mutation does not affect replication capacity when 2 T cell lines are used in an assay measuring it (46). Since a different effect of mutations on replication capacity

TABLE 3 Responses of CD8⁺ T cells from individuals infected with 3R virus to WT or 3R peptide

Patient ID	Virus sequence [mo/day/yr (type)]		PBMC sampling date (mo/day/yr)	PBMCs cultured with:	% IFN- γ -producing cells specific for each peptide among CD8 ⁺ T cells ^a		
	Early phase	Chronic phase			Without	WT	3R
KI-091	12/13/2000 (3R)	8/4/2005 (3R)	12/13/2000	WT	0.2	74.6	71.2
				3R	0.3	55.4	71.9
			9/29/2004	WT	0.2	77.7	65.5
KI-134	10/25/2001 (3R)	6/30/2004 (3R)	10/25/2001	WT	0.4	0.6	0.8
				3R	1.0	1.1	5.7
			1/21/2004	WT	0.8	1.0	0.7
KI-136	10/29/2001 (3R)	7/10/2003 (3R)	10/29/2001	WT	0.1	0.4	0.2
				3R	0.1	0.2	0.2
			5/15/2003	WT	0.4	0.8	0.4
KI-151	2/15/2002 (3R)	6/16/2005 (3R)	11/21/2001	WT	0.3	0.7	0.8
				3R	0.7	0.6	10.8
			7/28/2004	WT	0.4	0.7	1.3
KI-163	8/30/2002 (3R)	9/27/2004 (3R)	8/30/2002	WT	0.2	0.3	0.2
				3R	0.2	0.4	0.2
			8/29/2005	WT	0.3	0.5	0.2
				3R	0.4	0.6	6.9

^a Without, without peptide. Boldface, positive IFN- γ -producing response.

between cell lines and CD4⁺ T cells from a healthy individual is known (23), we measured the replication capacity of the 3R virus by using CD4⁺ T cells from a healthy individual. The results confirm that this mutation did not affect the replication capacity (Fig. 6), suggesting that the 3R mutant could not revert in HLA-A*24:02⁻ individuals.

DISCUSSION

It is known that CTLs recognizing escape mutants are elicited after the escape mutant had been selected by WT epitope-specific CTLs (2, 4, 12, 15, 33, 39) or in new escape mutant virus-infected hosts having the same restricted HLA allele (15). However, since the CTLs recognizing escape mutants have been not well analyzed, the role of these CTLs in the control of HIV-1 infections remains unclear. In the present study, we investigated 2 groups, HLA-A*24:02⁺ individuals infected with WT virus and those infected with 3R escape mutant virus. We found that both WT-specific and cross-reactive CD8⁺ T cells were elicited in individuals infected with WT virus. Interestingly, cross-reactive T cells had been elicited before the emergence of the 3R escape mutant virus, though a similar finding was made in previous studies that analyzed other epitope-specific CTLs (18, 25, 26, 34). The present study shows that WT-specific CD8⁺ T cells were predominantly elicited in an early phase of the infection and that the number of cross-reactive CD8⁺ T cells increased in the chronic phase. The CTL clones from early and chronic phases in KI-161 showed similar abilities to kill WT virus-infected or 3R virus-infected cells and activities to suppress both viruses, suggesting that cross-reactive CD8⁺ T cells elicited at the early phase were expanded via antigen presentation by 3R virus-infected cells at the chronic phase.

WT-specific and cross-reactive CTL clones from KI-092 and KI-161 at an early phase of the infection effectively killed WT-virus-infected cells and suppressed the replication of the WT vi-

rus, whereas they exhibited no and weak ability, respectively, to suppress that of the 3R virus. Cross-reactive CTL clones had the same ability to suppress the replication of WT virus as did the WT-specific CTL clones. These results strongly suggest that both CTLs selected the 3R virus in these individuals infected with the WT virus. The 3R virus was not selected within at least 1 year after Gag28-specific CTLs had been detected in the individuals infected with the WT virus. This finding indicates that the 3R mutation was more slowly selected by these CTLs than escape mutants selected at an acute phase of the infection (16, 19, 34, 44, 45). On the other hand, a previous study suggested that acute accumulation of mutations in this epitope occurs after an HIV-1 infection (6). However, the data shown in that study concerned mutations contained at position 1 of the epitope. In addition, those data may have included cases in which the individuals had been infected with the 3R mutant virus, because it may be assumed that 3R virus had accumulated in the cohorts analyzed. Cross-reactive CTL clones established from PBMCs at both early and chronic phases of KI-161 killed 3R virus-infected cells, though the killing activity against the 3R virus-infected cells was weaker than that against the WT virus-infected cells. These CTL clones weakly suppressed the replication of the 3R virus (Fig. 3C). This weak ability to suppress it might have delayed the emergence of the 3R mutation in these patients.

WT-specific CTLs were not induced by stimulation of early- or chronic-phase PBMCs from the 5 individuals in which the 3R mutation had been detected at the early phase with WT peptides. This finding supports the possibility that these individuals had been infected with the 3R virus. Only KI-091 had cross-reactive T cells at early and chronic phases of the infection. All CTL clones established from this patient had cross-reactivity, implying that the patient had been infected with WT virus and that 3R had been selected at an early phase. However, WT-specific CTL clones were not established from this patient. In addition, the cross-reactive

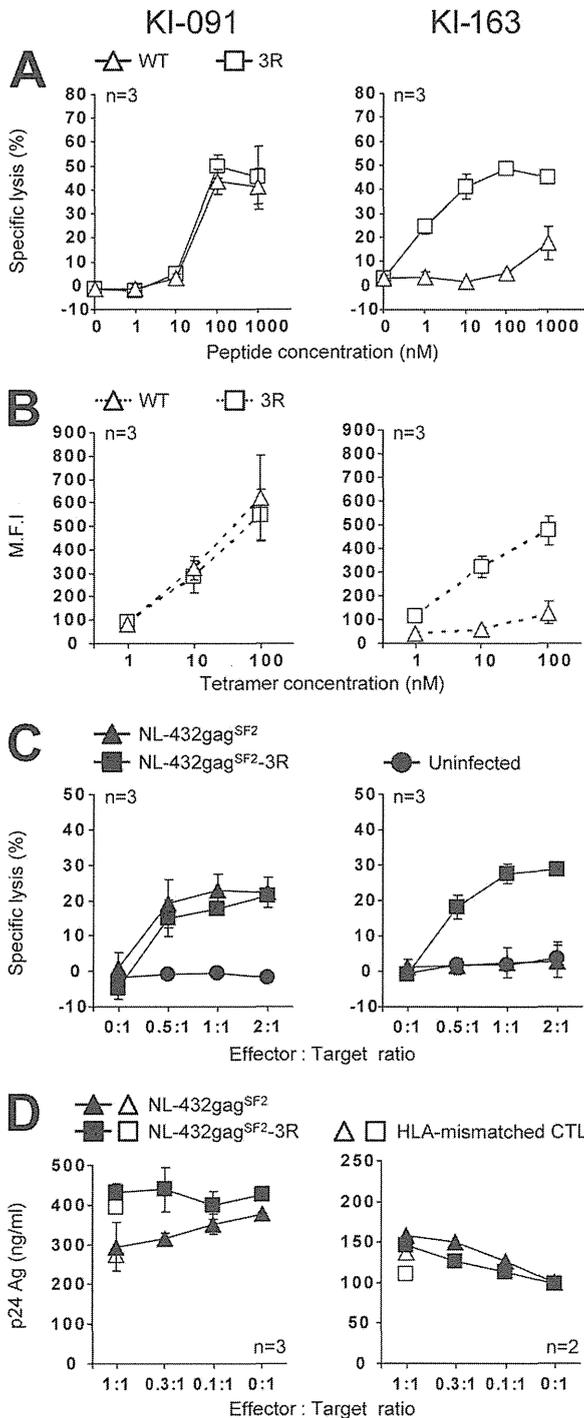


FIG 4 Antiviral activities of cross-reactive and 3R-specific CTL clones generated from patients KI-091 and KI-163 infected with 3R virus. Gag28-specific CTL clones were generated from chronic-phase PBMCs isolated from patients KI-091 and KI-163 after their stimulation with 3R peptide. The following activities of these CTL clones were analyzed. (A) Cytotoxic activity against 721.221-CD4-A2402 cells prepulsed with the WT or 3R peptide at concentrations of 1 to 1,000 nM. The cytotoxic activity was measured at an E:T ratio of 1:1. (B) Binding affinity toward WT and 3R tetramers at concentrations of 1 to 100 nM. The MFIs of the T cell clones are shown. (C) Cytotoxic activity against 721.221-CD4-A2402 cells infected with WT virus or 3R virus. WT-virus-infected and 3R virus-infected cells were used as target cells. The frequency of p24 Ag⁺ cells among the HIV-1-infected cells was as follows: WT-virus-infected cells, 49.1% and 43.1% for CTL clones from KI-091 and

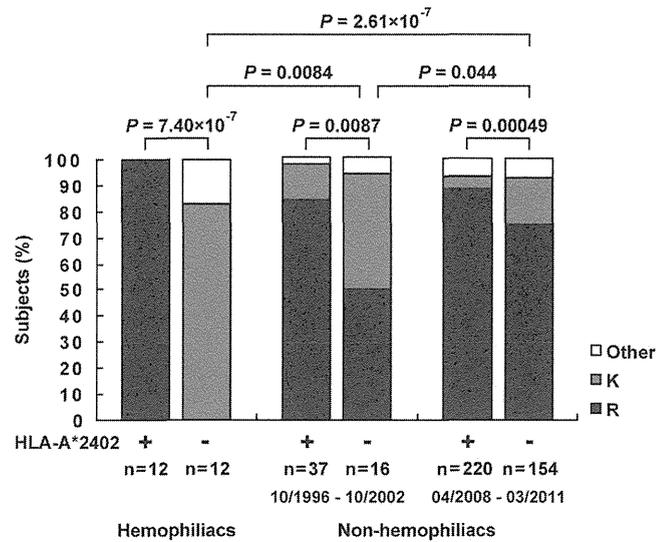


FIG 5 Frequencies of the 3R mutation in a Japanese hemophiliac cohort and nonhemophiliac cohorts recruited from 1996 to 2002 and from 2008 to 2011. The frequencies of mutations at position 3 of the Gag28 epitope in chronically HIV-1-infected HLA-A*24:02⁺ or HLA-A*24:02⁻ hemophiliac individuals and nonhemophiliac individuals recruited from 1996 to 2002 or from 2008 to 2011 are shown. The consensus sequence of this epitope in HIV-1 subtype B is KYKLVKLVW. The frequency of the 3R mutation between HLA-A*24:02⁺ and HLA-A*24:02⁻ subjects in each cohort or that in HLA-A*24:02⁺ or HLA-A*24:02⁻ subjects among the 3 cohorts was statistically analyzed by using Fisher's exact test.

CTL clones established from KI-091 did not have the ability to suppress the replication of the WT virus, although the CTL clones from individuals who had been infected with the WT virus had strong ability to suppress it. These findings suggest that this patient had been infected with the 3R virus rather than with the WT virus. However, it remains unknown why 3R-specific CTLs were elicited in the other 4 individuals but not in this patient. Thus, the abilities of CTLs to respond to WT peptide and to suppress the replication of WT virus together supported the idea that the individuals who had 3R virus in the early phase had been infected with 3R virus, although the possibility that they had been infected with WT virus cannot be completely excluded.

The 3R mutant epitope peptide would have been processed and presented to 3R-specific CTLs in 3R virus-infected cells, since 3R-specific and cross-reactive CTL clones effectively killed 3R virus-infected cells. However, these CTL clones failed to suppress the replication of the 3R virus. 721.221-CD4-A2402 cell lines were used as target cells for the killing assay, whereas CD4⁺ T cells from healthy individuals were used for the replication suppression assay. The former cells express HLA-A*24:02 to a much higher degree than the latter cells. This difference between the 2 cell lines may account for the discrepancy of the results between the 2 assays. 3R-specific CTL clones failed to suppress the replication of the 3R virus, whereas cross-reactive CTLs from the individuals

KI-163, respectively, and 3R-virus-infected cells, 48.6% and 45.6% for CTL clones from KI-091 and KI-163, respectively. The cytotoxic activity was measured at E:T ratios of 0.5:1, 1:1, and 2:1. (D) Abilities of the clones to suppress the replication of WT or 3R virus. The abilities were tested at different E:T ratios. n, number of clones tested. The error bars indicate standard deviations.

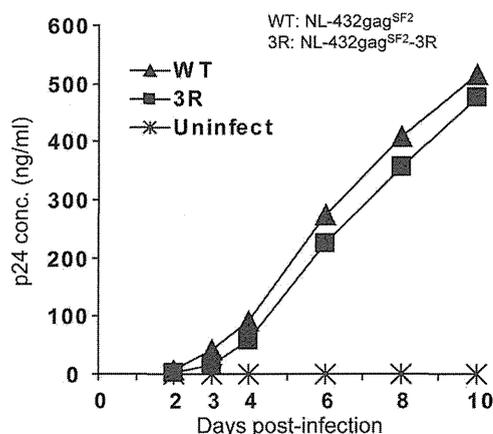


FIG 6 Replication kinetics of WT and 3R viruses in CD4⁺ T cells. CD4⁺ T cells (2×10^5) isolated from PBMCs from a healthy donor were infected with WT or 3R virus in triplicate at a blue-cell-forming unit of 500 (in MAGIC-5 cells) in a total volume of 0.2 ml and then incubated at 37°C for 2 h. The infected cells were washed twice with R10 and then cultured in 1 ml of complete medium plus rIL-2 at 37°C. A 0.1-ml volume of the culture supernatants was collected at days 2 to 10 postinfection. The concentration of p24 Ag was measured by using ELISA.

infected with WT virus effectively suppressed the replication of the WT virus but failed to suppress that of the 3R virus. These findings suggest that 3R virus-infected CD4⁺ T cells could not effectively present the 3R mutant epitope. This finding also suggests that 3R virus-infected CD4⁺ T cells were not the main source of antigen-presenting cells in 3R virus-infected individuals. A previous study showed that HIV-1-infected macrophages effectively present HIV-1 epitopes more than HIV-1-infected CD4⁺ T cells (14), implying that 3R virus-infected macrophages are the main antigen-presenting cells and contribute to the elicitation of 3R-specific and cross-reactive CTLs in 3R virus-infected individuals. A further study should clarify the role of macrophages in the elicitation of 3R-specific and cross-reactive CTLs in 3R virus-infected individuals.

Cross-reactive CTLs were found in individuals infected with the WT virus or with the 3R virus. The CTL clones established from individuals infected with the WT virus had a strong ability to kill WT-virus-infected cells and to suppress the replication of the WT virus, whereas those established from an individual infected with the 3R virus showed moderate ability to kill WT-virus-infected cells and no ability to suppress the replication of WT virus. These findings indicate that cross-reactive CTLs from an individual infected with the 3R virus may have had less ability to recognize the WT epitope than those from an individual infected with the WT virus. Indeed, the former CTL clones exhibited lower sensitivity to reaction with WT peptide-pulsed cells than the latter CTLs, indicating that cross-reactive CTLs elicited in individuals infected with the WT virus had higher-affinity TCRs for WT peptide than those in an individual infected with the 3R virus. In addition, the latter CTL clones weakly killed 3R virus-infected cells, whereas the former clones showed the same killing activity against 3R virus-infected cells as against WT-virus-infected cells. Thus, cross-reactive CTLs in individuals infected with 3R virus have different characteristics than those in individuals infected with the WT virus. This finding suggests that cross-reactive CTLs elicited in individuals infected with the WT virus had TCRs with higher affinity for WT and 3R peptides than those in individuals infected with the 3R virus.

Japanese hemophiliacs were infected with HIV-1 via blood products from the United States around 1983, and HLA-A*24:02 is a rare allele in North America. Therefore, it may be speculated that HIV-1 in the blood product had not yet accumulated escape mutations. Indeed, the 3R mutation was not found in the 12 HLA-A*24:02⁻ hemophiliacs tested, though other amino acid variants at position 3 were detected in 2 of these hemophiliacs. This mutation was found in 50.0% of HLA-A*24:02⁻ individuals in the 1996 to 2002 cohort and in 74.7% of those in the 2008 to 2011 cohort, indicating that the mutation had accumulated in the Japanese population. The frequency of this mutation in HLA-A*24:02⁻ individuals thus increased about 1.5-fold during the approximately 10-year period between these 2 nonhemophiliac cohorts. Thus, the mutation greatly accumulated over the last 10 years. Since HLA-A*24:02 is found in approximately 70% of Japanese, the high prevalence of the allele is the cause of the high accumulation of the 3R mutation in the Japanese population. In addition, this high accumulation resulted not only from a strong selection of the 3R mutation by WT-specific and cross-reactive CTLs elicited in the donors infected with WT virus, but also from a lack of reversion of the mutation in the HLA-A*24:02⁻ individuals.

Our previous study concerning HLA-A*24:02-restricted Nef138-specific CTLs demonstrated that only WT epitope-dominant CTLs, which suppress the replication of WT virus but fail to suppress that of mutant virus, are elicited at an early phase in HLA-A*24:02⁺ individuals infected with the WT virus and that mutant-epitope-dominant CTLs but not cross-reactive CTLs are elicited after the emergence of the mutant virus in them (15). In addition, only mutant-epitope-dominant CTLs are elicited in those individuals infected with the mutant virus. The mutant-epitope-dominant CTLs suppress the replication of WT virus but weakly suppress that of mutant virus (15). Thus, Nef138-specific CTLs elicited in individuals infected with WT or mutant viruses had different characteristics in terms of the recognition of WT and mutant epitopes than the Gag28-specific CTLs analyzed in the present study. The difference between Nef138-specific and Gag28-specific CTLs might be explained by a different CTL repertoire elicited at an early phase. These 2 studies suggest the elicitation of various HIV-1-specific CTLs in regard to recognition of escape mutations.

In the present study, we demonstrated that WT-specific and cross-reactive CTLs were elicited at an early phase in individuals infected with the WT virus and that cross-reactive CTLs were dominant in Gag28-specific CTLs after the emergence of the 3R virus. On the other hand, 3R-specific and cross-reactive CTLs were elicited in individuals infected with the 3R virus, though the former CTLs were predominantly elicited in these individuals. The CTLs elicited in the individuals infected with the WT virus, which had a strong ability to suppress the replication of WT virus, played a central role in the accumulation of the 3R mutation. In contrast, the CTLs elicited in those infected with 3R virus, which failed to suppress the replication of WT and 3R viruses, did not contribute to the control of the 3R virus infection. In addition, the high prevalence of HLA-A*24:02 and lack of effect of the 3R mutation on viral fitness may have strongly contributed to the high accumulation of the mutation in HIV-1-infected Japanese individuals.

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Renal Function Declines More in Tenofovir- than Abacavir-Based Antiretroviral Therapy in Low-Body Weight Treatment-Naïve Patients with HIV Infection

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Abstract

Objective: To compare the rate of decline of renal function in tenofovir- and abacavir-based antiretroviral therapy (ART) in low-body weight treatment-naïve patients with HIV infection.

Design: We conducted a single-center retrospective cohort study of 503 Japanese patients who commenced on either tenofovir- or abacavir-based initial ART.

Methods: The incidence of renal dysfunction, defined as more than 25% fall in estimated glomerular filtration rate (eGFR) from the baseline, was determined in each group. The effect of tenofovir on renal dysfunction was estimated by univariate and multivariate Cox hazards models as the primary exposure. Changes in eGFR until 96 weeks were estimated in both groups with a repeated measures mixed model.

Results: The median body weight of the cohort was 64 kg. The estimated incidence of renal dysfunction in the tenofovir and the abacavir arm was 9.84 per 100 and 4.55 per 100 person-years, respectively. Tenofovir was significantly associated with renal dysfunction by univariate and multivariate analysis (HR=1.747; 95% CI, 1.152–2.648; p=0.009) (adjusted HR=2.080; 95% CI, 1.339–3.232; p<0.001). In subgroup analysis of the patients stratified by intertertile baseline body weight, the effect of tenofovir on renal dysfunction was more evident in patients with lower baseline body weight by multivariate analysis (≤ 60 kg: adjusted HR=2.771; 95%CI, 1.494–5.139; p=0.001) (61–68 kg: adjusted HR=1.908; 95%CI, 0.764–4.768; p=0.167) (> 68 kg: adjusted HR=0.997; 95%CI, 0.318–3.121; p=0.995). The fall in eGFR was significantly greater in the tenofovir arm than the abacavir arm after starting ART (p=0.003).

Conclusion: The incidence of renal dysfunction in low body weight patients treated with tenofovir was twice as high as those treated with abacavir. Close monitoring of renal function is recommended for patients with small body weight especially those with baseline body weight <60 kg treated with tenofovir.

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Introduction

Tenofovir disoproxil fumarate (TDF) and abacavir sulfate (ABC) are widely used nucleot(s)ide reverse transcriptase inhibitors (NRTIs) as part of the initial antiretroviral therapy for patients with HIV infection in the developed countries (URL:<http://www.aidsinfo.nih.gov/ContentFiles/AdultandAdolescentGL.pdf>) (URL:http://www.europeanclinicalaidsociety.org/images/stories/EACS-Pdf/1_treatment_of_hiv_infected_adults.pdf). TDF is generally preferred to ABC, since ABC is reported to cause serious hypersensitivity

reaction in 5–8% of the patients and its efficacy in viral suppression is reported to be inferior to TDF among patients with baseline HIV viral load of >100,000 copies/ml [1,2]. On the other hand, renal proximal tubular damage and renal dysfunction are well-known adverse effects of TDF [3–9]. A meta-analysis study that compared TDF and other NRTIs concluded that the decline in renal function with TDF use is significant but modest, and the ASSERT study conducted in Europe compared randomly-selected treatment naïve patients who commenced treatment with either TDF or ABC with efavirenz and showed no difference in estimated glomerular filtration

rate (eGFR) between the two groups at 48 weeks [9,10]. To date, the nephrotoxicity of TDF have been regarded as mild and tolerable [2,5–7,9–11].

However, the TDF-related nephrotoxicity has hardly been evaluated in patients with small body weight, who are potentially at higher risk for larger drug exposure and thus, more severe toxicity [12–15]. Indeed, some recent studies including ours reported a higher incidence of TDF-related renal dysfunction among Asian patients with low body weight compared with previous studies on mostly Whites and African Americans with larger body weight [13,16]. Thus, it is important to provide more evidence in support of TDF-associated nephrotoxicity in patients with low body weight since such data can elucidate whether TDF-related nephrotoxicity is as mild in low-body-weighted patients as previously reported in Europe and the USA. This is also important because there is increasing use of TDF in resource-limited settings, where patients are often of relatively small body weight, following the revised 2010 WHO guidelines that recommend TDF as one of the components of first line therapies (URL:http://whqlibdoc.who.int/publications/2010/9789241599764_eng.pdf) [13,16–19]. To our knowledge, there are no studies that compared renal function in treatment naïve Asian patients who commenced treatment with TDF or ABC.

Based on the above background, the present study was designed to compare the incidence of renal dysfunction and change in eGFR between treatment-naïve Japanese patients with low body weight who started either TDF or ABC as part of the antiretroviral regimen.

Methods

Ethics Statement

This study was approved by the Human Research Ethics Committee of National Center for Global Health and Medicine, Tokyo. All patients included in this study provided a written informed consent for their clinical and laboratory data to be used and published for research purposes. This study has been conducted according to the principles expressed in the Declaration of Helsinki.

Study Subjects

We performed a retrospective, single-center cohort study of HIV-infected Japanese patients using the medical records at the National Center for Global Health and Medicine, Tokyo, Japan. Our facility is one of the largest clinics for patients with HIV infection in Japan with more than 2,700 registered patients. The study population was treatment-naïve patients with HIV infection, aged >17 years, who commenced treatment with either the recommended 300 mg/day dose of TDF or 600 mg/day dose of ABC-containing antiretroviral regimen at our clinic between January 1, 2004 and March 31, 2009. During this inclusion period, all except two patients at our clinic started ART with either ABC or TDF. Patients with an eGFR of >60 ml/min/1.73 m² were enrolled. Patients were followed up until March 31, 2011. They were excluded if they started ART with both TDF and ABC, their follow-up period at our facility was less than 24 weeks after commencement of ART, or if they had started ART at other facilities. Only Japanese patients were included in order to examine a population with comparatively homogenous basic demographics and background. The attending physician selected either TDF or ABC at baseline, and the use of these two drugs was based on the Japanese guidelines, which place both ABC and TDF as the preferred NRTIs (<http://www.haart-support.jp/guideline2011.pdf>, in Japanese). The attending physician also selected

the key drug [non-nucleoside reverse transcriptase inhibitor (NNRTI), protease inhibitor (PI), or integrase inhibitor (INI)]. All patients received standard ART with two NRTIs combined with either PI, NNRTI, or INI.

Measurements

We defined renal dysfunction as more than 25% decrease in eGFR relative to the baseline [13,16,20,21]. The baseline eGFR was estimated for each patient from the average of two successive serum creatinine measurements made closest to and preceding the commencement of antiretroviral therapy by no more than 90 days. Changes in eGFR were plotted from the baseline measurement until the average value of two successive measurements diminished to less than 75% of the baseline, discontinuation of TDF or ABC, or at the end of the follow-up period. Discontinuation of TDF and ABC was the choice of the attending physician, and was based on virologic failure or ART-related side effects other than renal dysfunction. Before the initiation of ART and until suppression of HIV-1 viral load, patients visited our clinic every month. However, after viral load suppression, the visit interval was extended up to every three months. Serum creatinine and eGFR were measured in every visit, and the frequency of measurements was similar in patients on TDF and ABC. eGFR was calculated using the equation from the 4-variable Modification of Diet in Renal Disease (MDRD) study, $eGFR = 186 \times [\text{serum creatinine}]^{-1.154} \times [\text{age}]^{-0.203} \times [0.742 \text{ if patient is female}] \times [1.212 \text{ if patient is African American}]$ [22]. In this study, the primary exposure variable was TDF use over ABC as part of the initial ART.

The potential risk factors for renal dysfunction were determined according to previous studies and collected together with the basic demographics from the medical records [15,23–25]. They included age, sex, body weight, body mass index, (BMI) = {body weight (kg) / [(height (m))²]}, baseline laboratory data (CD4 cell count, HIV viral load, and serum creatinine), and presence or absence of other medical conditions (concurrent use of ritonavir-boosted protease inhibitors, concurrent nephrotoxic drugs such as ganciclovir, sulfamethoxazole/trimethoprim, and non-steroidal anti-inflammatory agents, diabetes mellitus defined by using anti-diabetic agents or fasting plasma glucose >126 mg/dl or plasma glucose >200 mg/dl on two different days, co-infection with hepatitis B defined by positive hepatitis B surface antigen, co-infection with hepatitis C defined by positive HCV viral load, hypertension defined by current treatment with antihypertensive agents or two successive measurements of systolic blood pressure >140 mmHg or diastolic blood pressure >90 mmHg at the clinic, dyslipidemia defined by current treatment with lipid-lowering agents, and current smoking). At our clinic, weight and blood pressure were measured on every visit whereas other variables were measured in the first visit and at least once annually. We used the data on or closest to and preceding the day of starting ART by no more than 90 days.

Statistical analysis

The time to 25% decline in eGFR from the baseline was calculated from the date of commencement of treatment to the date of diagnosis of the above-defined renal dysfunction. Censored cases represented those who discontinued ABC or TDF, dropped out, were referred to other facilities, or at the end of follow-up period. The time from the start of ART to >25% decrease in eGFR was analyzed by the Kaplan Meier method for patients who started TDF (TDF arm) and ABC (ABC arm), and the log-rank test was used to determine the statistical significance. The Cox proportional hazards regression analysis was used to estimate the

impact of TDF use over ABC on the incidence of more than 25% decrease in eGFR relative to the baseline. The impact of each basic demographics, baseline laboratory data, and other medical conditions listed above was also estimated with univariate Cox proportional hazards regression.

To estimate the unbiased prognostic impact of TDF use over ABC for renal dysfunction, we conducted three models using multivariate Cox proportional hazards regression analysis. Model 1 was the aforementioned univariate analysis for TDF use over ABC. Model 2 included age and weight plus model 1 in order to adjust for basic characteristics. In model 3, we added variables with P values <0.05 in univariate analysis for adjustment (these included age per 1 year, weight per 1 kg decrement, CD4 count per 1 μl decrement, HIV viral load per \log_{10}/ml , serum creatinine per 1 mg/dl , concurrent use of nephrotoxic drug(s), hepatitis B infection, and diabetes mellitus). The eGFR and the BMI were excluded from multivariate analysis because of their multicollinearity with age and serum creatinine, and weight, respectively, since eGFR and BMI are gained by the equation of those variables [22,26]. We chose to add weight instead of BMI because our previous work showed that weight was more useful and handy information to estimate the risk for TDF-related nephrotoxicity than BMI [16].

As a sensitivity analysis, creatinine clearance was similarly calculated with Cockcroft-Gault equation for each patient, creatinine clearance = $[(140 - \text{age}) \times \text{weight (kg)}] / (\text{serum creatinine} \times 72) (\times 0.85 \text{ for females})$ [27]. Actual body weight was used for the calculation. The impact of TDF use over ABC for $>25\%$ decrement of creatinine clearance from the baseline was estimated in univariate analysis and multivariate analysis adjusted with the before mentioned variables with Cox proportional hazards model.

To estimate the impact of weight on TDF-related nephrotoxicity, we did subgroup analysis for intertertile baseline body weight categories: ≤ 60 , 61–68, and >68 kg. Then, the abovementioned multivariate analysis with eGFR was conducted for each subgroup.

We also used a repeated measures mixed model to estimate and compare changes in eGFR between ABC and TDF from baseline to 2 years after initiation of ART by 6-month intervals adjusted for baseline eGFR and weight [10]. For each patient, the eGFR values at closest to and preceding 24, 48, 72 and 96 weeks after commencement of ART were collected. In this analysis, censoring occurred at discontinuation of TDF or ABC, leaving care, or reaching the end of the observation period before 96 weeks. Sensitivity analysis with creatinine clearance calculated by Cockcroft-Gault equation was similarly conducted.

Statistical significance was defined at two-sided p values <0.05 . We used hazard ratios (HRs) and 95% confidence intervals (95% CIs) to estimate the impact of each variable on renal dysfunction. All statistical analyses were performed with The Statistical Package for Social Sciences ver. 17.0 (SPSS, Chicago, IL).

Results

The study subjects were 199 patients in the TDF arm and 304 patients in the ABC arm who fulfilled the abovementioned criteria. Table 1 shows the demographics, laboratory data, and medical conditions of the study population at baseline. The majority of the study population was males, comparatively young and had a small stature (median weight, 64 kg, median BMI, $22.2 \text{ kg}/\text{m}^2$). More than 80% of the patients in the two arms had ritonavir-boosted PI. In the ABC arm, patients had significantly lower CD4 count ($p=0.006$), were significantly more likely to have hypertension

($p<0.001$), and tended to use more nephrotoxic drugs ($p=0.109$). On the other hand, in the TDF arm, patients had marginally higher baseline eGFR ($p=0.098$) and were significantly more likely to have hepatitis B virus infection ($P<0.001$). However, all other major background parameters were similar in the two groups (Table 1).

More than 25% decrement in eGFR from baseline occurred in 44 patients (22.1%) in the TDF arm and 41 (13.5%) in the ABC arm, with an estimated incidence of 9.84 and 4.55 per 100 person-years, respectively. Figure 1 shows the time from ART initiation to $>25\%$ decrease in eGFR by the Kaplan Meier method in the two groups. Patients who started TDF-containing ART were significantly more likely to develop renal dysfunction, compared to the ABC group ($p=0.001$, Log-rank test). The median time from commencement of ART to occurrence of $>25\%$ decrement in eGFR was 246 days (range, 1–1,339 days) for the TDF arm and 501 days (range, 7–2,022) for ABC arm. The total observation period was 447.2 patient-years [median, 839 days, interquartile range (IQR), 357–1137 days] for the TDF arm and 901.7 patient-years (median, 1,119 days, IQR, 660.5–1509 days) for the ABC arm.

Univariate analysis showed a significant relationship between TDF use and $>25\%$ decrement in eGFR (HR = 1.747; 95%CI, 1.152–2.648; $p=0.009$) (Table 2). Furthermore, old age, small body weight, low baseline CD4 count, high HIV viral load, high eGFR, low serum creatinine, concurrent use of nephrotoxic drugs, hepatitis B infection, and diabetes mellitus were associated with renal dysfunction. On the other hand, concurrent use of ritonavir boosted PIs was not associated with renal dysfunction (HR = 1.220; 95%CI, 0.663–2.244; $p=0.523$). Multivariate analysis identified TDF use as a significant risk for $>25\%$ decrement in eGFR after adjustment for age and weight (adjusted HR = 1.893; 95%CI, 1.243–2.881; $p<0.003$) (Table 3, Model 2), and also after adjustment for other risk factors (adjusted HR = 2.080; 95%CI, 1.339–3.232; $p<0.001$) (Table 3, Model 3). We also conducted a sensitivity analysis using BMI decrement instead of weight as a variable in Table 3, Model 3. The results were almost identical; TDF use over ABC use was a risk for renal dysfunction (adjusted HR 1.957, 95% CI 1.262–3.036, $p=0.003$).

Sensitivity analysis with creatinine clearance confirmed the abovementioned findings: both univariate and multivariate analyses showed that TDF use was significantly associated with $>25\%$ decrement in eGFR (univariate analysis: HR = 2.212; 95%CI, 1.340–3.653; $p=0.002$) (multivariate analysis: adjusted HR = 2.544; 95%CI, 1.493–4.335; $p=0.001$).

Subgroup analysis of the patients stratified by intertertile baseline body weight showed that the lower the baseline body weight, the more evident the impact of TDF on renal dysfunction (≤ 60 kg: adjusted HR = 2.771; 95%CI, 1.494–5.139; $p=0.001$) (61–68 kg: adjusted HR = 1.908; 95%CI, 0.764–4.768; $p=0.167$) (>68 kg: adjusted HR = 0.997; 95%CI, 0.318–3.121; $p=0.995$) (Table 4). These findings suggest that there is the effect modification by baseline body weight on TDF-associated renal dysfunction.

Data analysis by repeated measures mixed models showed a significant decrease in adjusted mean eGFR from the baseline to 96 weeks in both groups (TDF: $-9.984 \text{ ml}/\text{min}/1.73\text{m}^2$, 95%CI -12.05 to $-7.914 \text{ ml}/\text{min}/1.73\text{m}^2$, $p<0.001$; ABC: $-5.393 \text{ ml}/\text{min}/1.73\text{m}^2$, 95%CI -7.087 to $-3.699 \text{ ml}/\text{min}/1.73\text{m}^2$, $p<0.001$) (Figure 2). There was a statistically significant interaction between the two arms over time ($p=0.003$), indicating that adjusted mean eGFR decreased more significantly in the TDF group than in the ABC group after initiation of ART. Analysis of eGFR in each group demonstrated a rapid decrease during the first 24 weeks,

Table 1. Baseline demographics and laboratory data of patients who received tenofovir- and abacavir-based antiretroviral therapy (n = 503).

	TDF (n = 199)	ABC (n = 304)	P value
Sex (male), n (%)	196 (98.5)	296 (97.4)	0.539
Median (IQR) age	36 (31–44)	37 (31–43)	0.436
Median (IQR) weight (kg)	64 (58–69)	64 (58.0–70.9)	0.426
Median (IQR) BMI (kg/m ²)	22.1 (20.4–23.9)	22.2 (20.3–24.6)	0.321
Median (IQR) eGFR (ml/min/1.73m ²)	119.4 (103.0–135.0)	115.6 (102.4–132.2)	0.098
Median (IQR) serum creatinine (mg/dl)	0.74 (0.67–0.84)	0.75 (0.68–0.83)	0.250
Median (IQR) CD4 count (/μl)	199 (109–272)	178.5 (75.3–234.8)	0.006
Median (IQR) HIV RNA viral load (log ₁₀ /ml)	4.63 (4.20–5.20)	4.74 (4.23–5.20)	0.731
Ritonavir-boosted protease inhibitors, n (%)	173 (86.9)	256 (84.2)	0.441
Protease inhibitors (unboosted), n (%)	5 (2.5)	20 (6.6)	0.038
NNRTIs, n (%)	16 (8.0)	26 (8.6)	0.848
INIs, n (%)	5 (2.5)	2 (0.7)	0.119
Hypertension, n (%)	5 (2.5)	53 (17.4)	<0.001
Dyslipidemia, n (%)	4 (2.0)	4 (1.3)	0.718
Diabetes mellitus, n (%)	8 (4.0)	12 (3.9)	1.000
Concurrent use of nephrotoxic drugs, n (%)	65 (32.7)	121 (39.8)	0.109
Hepatitis B, n (%)	35 (17.6)	9 (3.0)	<0.001
Hepatitis C, n (%)	7 (3.5)	7 (2.3)	0.421
Current smoker, n (%)	93 (46.7)	149 (49.3)	0.585

TDF: tenofovir, ABC: abacavir, IQR: interquartile range, BMI: body mass index, eGFR: estimated glomerular filtration rate, NNRTI: non- nucleoside reverse transcriptase inhibitor, INI: integrase inhibitor.

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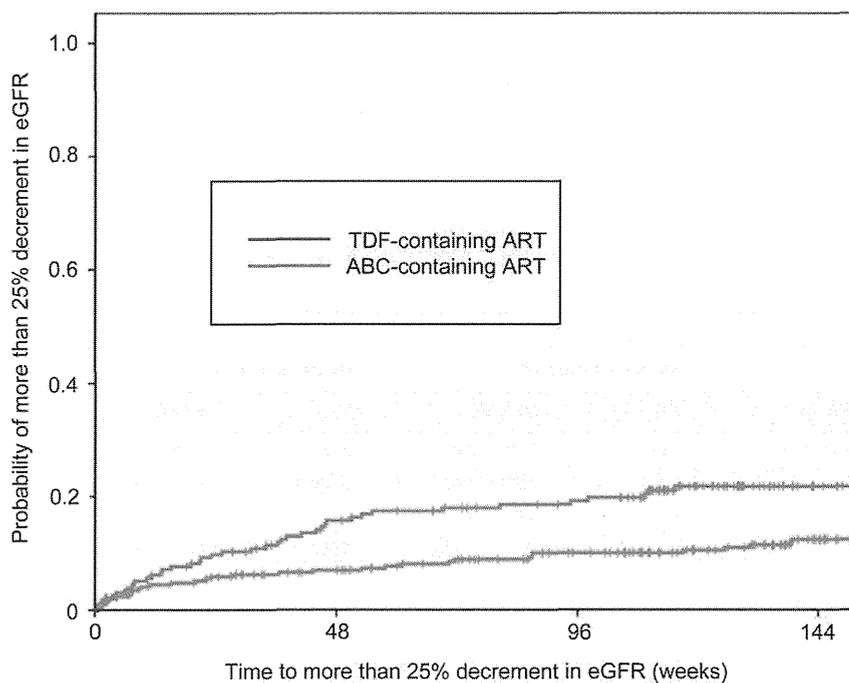


Figure 1. Kaplan-Meier curve showing the time to renal dysfunction in patients treated with TDF or ABC. Compared to treatment-naïve patients who commenced treatment with ABC, those on TDF were more likely to develop >25% fall in eGFR (p = 0.001, Log-rank test). TDF: tenofovir, ABC: abacavir, ART: antiretroviral therapy, eGFR: estimated glomerular filtration rate.

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Table 2. Univariate analysis to estimate the risk of various factors in inducing more than 25% fall in eGFR.

	Hazard ratio	95% CI	P value
TDF vs. ABC use	1.747	1.152–2.648	0.009
Female gender	0.048	0.000–16.93	0.310
Age per 1 year	1.031	1.011–1.051	0.002
Weight per 1 kg decrement	1.047	1.023–1.072	<0.001
BMI per 1 kg/m ² decrement	1.152	1.066–1.244	<0.001
CD4 count per 1 /μl decrement	1.006	1.004–1.008	<0.001
HIV viral load per log10/ml	1.562	1.179–2.071	0.002
Ritonavir-boosted protease inhibitors	1.220	0.663–2.244	0.523
Baseline eGFR per 1 ml/min/1.73m ²	1.009	1.005–1.014	<0.001
Baseline serum creatinine per 1 mg/dl	0.016	0.003–0.086	<0.001
Concurrent nephrotoxic drug	2.134	1.417–3.214	<0.001
Hepatitis B	1.866	1.038–3.356	0.037
Hepatitis C	1.721	0.631–4.695	0.289
Diabetes mellitus	2.558	1.181–5.540	0.017
Hypertension	0.865	0.448–1.669	0.664
Current smoking	0.989	0.657–1.489	0.958

eGFR: estimated glomerular filtration rate, CI: confidence interval, TDF: tenofovir, ABC: abacavir, BMI: body mass index.
doi:10.1371/journal.pone.0029977.t002

followed by a plateau until 96 weeks. In sensitivity analysis with creatinine clearance calculated by Cockcroft-Gault equation, the result was the same; a significant decrease from the baseline to 96 weeks in both groups (TDF: -10.62 ml/min, 95%CI -13.78 to -7.458 ml/min; ABC: -4.325 ml/min, 95%CI -6.893 to -1.756 ml/min) and significantly more eGFR decrement in the TDF group (p = 0.019).

Discussion

In this observational Japanese cohort, treatment-naïve patients who started TDF-containing ART experienced eGFR decline of >25% approximately twice as likely compared to those treated with ABC-containing regimen. Univariate and multivariate analyses identified TDF use as an independent risk factor for

Table 4. Multivariate analysis to estimate the risk of TDF-over ABC-based antiretroviral therapy in the induction of more than 25% fall in eGFR according to baseline body weight.

	Adjusted HR	95% CI	P value
Baseline body weight ≤60 kg (n = 171)			
TDF vs. ABC use	2.771	1.494–5.139	0.001
Baseline body weight 61–68 kg (n = 167)			
TDF vs. ABC use	1.908	0.764–4.768	0.168
Baseline body weight >68 kg (n = 165)			
TDF vs. ABC use	0.997	0.318–3.121	0.995

TDF use was adjusted with the same variables indicated in Model 3, Table 3: age per 1 year, weight per 1 kg decrement, CD4 count per 1 /μl decrement, HIV viral load per log10/ml, serum creatinine per 1 mg/dl, concurrent use of nephrotoxic drugs, hepatitis B infection, and diabetes mellitus.
doi:10.1371/journal.pone.0029977.t004

renal dysfunction. Subgroup analysis showed that the effect of TDF on renal dysfunction was more evident in patients with lower body weight. Furthermore, eGFR decrement was significantly larger in the TDF group than in ABC group over the 2-year observation period.

In our previous study, we demonstrated a high incidence of TDF-associated nephrotoxicity in patients with low body weight, and the use of a robust statistical model indicated a greater decline in renal function in patients of low body weight treated with TDF [16]. The results of the present study further emphasize the importance of low body weight as a risk factor for TDF-related nephrotoxicity by showing that in a cohort of patients with low body weight, the incidence of renal dysfunction was twice higher with TDF use than with ABC use.

Among the studies designed to compare renal function after the commencement of TDF and ABC-containing ART for treatment-naïve patients, our cohort had the lowest median body weight (64 kg). This is lower than the median body weight of patients of the ASSERT study conducted in European countries (72 kg) [10]. The

Table 3. Multivariate analysis to estimate the risk of TDF- over ABC-based antiretroviral therapy in inducing more than 25% fall in eGFR.

	Model 1 Crude		Model 2 Adjusted		Model 3 Adjusted	
	HR	95% CI	HR	95%CI	HR	95%CI
TDF vs. ABC use [†]	1.747	1.152–2.648	1.893	1.243–2.881	2.080	1.339–3.232
Age per 1 year			1.029	1.010–1.048	1.020	1.000–1.040
Weight per 1 kg decrement [†]			1.046	1.022–1.071	1.028	1.005–1.052
CD4 count per 1 /μl decrement [†]					1.004	1.002–1.007
HIV viral load per log10/ml					1.048	0.749–1.466
Serum creatinine per 1 mg/dl [†]					0.053	0.009–0.304
Use of nephrotoxic drug					1.309	0.825–2.077
Hepatitis B					1.070	0.573–2.000
Diabetes mellitus					1.565	0.684–3.582

[†]P<0.05 in Model 3.

TDF: tenofovir, ABC: abacavir, eGFR: estimated glomerular filtration rate, HR: hazard ratio, CI: confidence interval.

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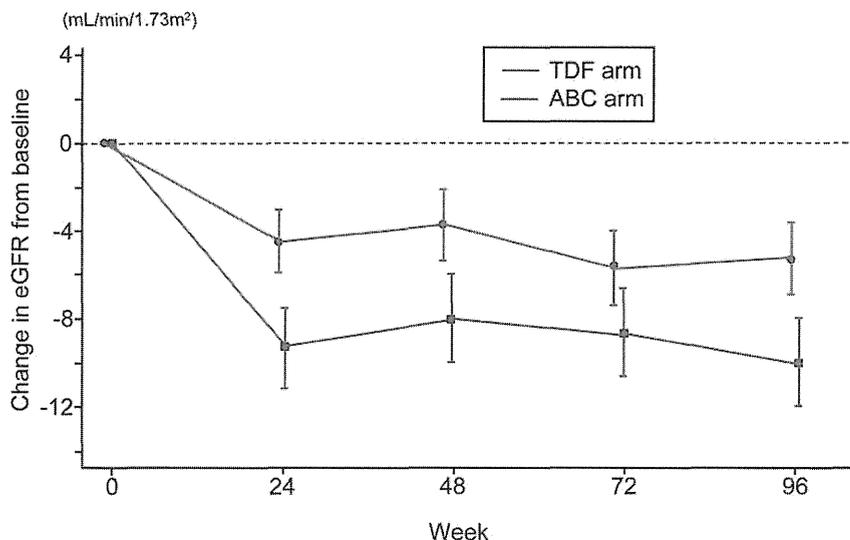


Figure 2. Changes in eGFR in patients treated with TDF or ABC between baseline and 96 weeks. The fall in eGFR was significantly greater in the TDF group than the ABC group ($p = 0.003$). Data are adjusted mean \pm 95% confidence interval. eGFR: estimated glomerular filtration rate, TDF: tenofovir, ABC: abacavir. doi:10.1371/journal.pone.0029977.g002

results of the present study on TDF-related nephrotoxicity differ from the findings of randomized clinical trials that demonstrated no major change in renal function of TDF- and ABC-treated patients over 48–96 week follow-up [2,10,11]. The discrepant results might arise from differences between observational cohort and clinical trials, since observational studies tend to express the results in “real world setting” whereas clinical trials include patients who fulfill more strict criteria, therefore with better profile [9]. The discrepant results could be also due to the use of different definitions for renal dysfunction in these studies. However, the discrepant results could also reflect the difference in median body weight between the present study and these clinical trials. The results of our subgroup analysis support this hypothesis by showing that the effect of TDF on renal dysfunction was more evident in patients with low body weight. Apart from being low-body-weighted, the patients in this study did not appear to have many of other established risks for TDF-related nephrotoxicity; they were comparatively young, had relatively stable CD4 count, and had only a few co-morbidities (Table 1). Although the majority concurrently used ritonavir-boosted PIs, which are a probable risk for TDF-related nephrotoxicity, ritonavir-boosted PIs were not significantly associated with renal dysfunction in our cohort (Table 2) [24].

Changes in eGFR in those patients treated with TDF-containing ART were characterized by a rapid decline during the first 24 weeks of therapy, followed by a plateau until 96 weeks (Fig. 2). This finding is consistent with that reported from the Johns Hopkins group [9,28]. Together with the finding that the median time from commencement of ART to the $>25\%$ decline in eGFR in the TDF-treated patients was 246 days, these results suggest that careful monitoring of renal function is particularly warranted in the first year of TDF-based therapy. Thus, we suggest that renal function should be monitored by measurement of serum creatinine at least once annually in resource-limited settings and twice annually in resource-rich settings in patients starting TDF-containing ART, especially those with baseline body weight <60 kg.

The Department of Health and Human Services guideline for the treatment of HIV infection in the U.S. lists ABC as an

alternative NRTI because it can potentially cause serious hypersensitivity reaction and cardiovascular diseases (URL:<http://www.aidsinfo.nih.gov/ContentFiles/AdultandAdolescentGL.pdf>). However, some international guidelines consider both TDF and ABC as the preferred NRTIs under the condition that ABC should be used with caution in patients with viral load $>100,000$ copies/mL, based on the low incidence of ABC-related hypersensitivity among HLA-B*5701-negative population and the controversial association between ABC and cardiovascular diseases [1,29–32] (URL: http://www.europeanaidscouncil.org/images/stories/EACS-Pdf/1_treatment_of_hiv_infected_adults.pdf) (<http://www.haart-support.jp/guideline2011.pdf> in Japanese). The present study, together with our previous analysis that demonstrated preferential TDF-related nephrotoxicity in patients with low body weight, emphasize the advantage of ABC over TDF with regard to prognosis of renal function in low body weight patients [16].

TDF is the prodrug of acyclic nucleotide analog tenofovir, which is excreted by both glomerular filtration and active tubular secretion. Tenofovir is considered to cause mitochondrial damage in proximal renal tubular cells [33]. The concentration of tenofovir in the proximal renal tubules could be augmented with the complex interactions of pharmacological, environmental, and genetic factors, including small body weight, consequently resulting in renal tubular dysfunction [34]. Body weight has been identified as an important factor in TDF-related nephrotoxicity not only in clinical trials, but also in *in vitro* and pharmacokinetic studies [35–37].

The present study has several limitations. First, because of its retrospective nature, it was not possible to control the baseline characteristics of the enrolled patients. Thus, it is possible that patients with potential risk for TDF-related nephrotoxicity were not prescribed TDF. A proportion of patients treated with ABC had low CD4 count and others were hypertensive, both conditions are known risk factors for renal dysfunction [23,25]. However, for these reasons, the incidence of TDF-associated renal dysfunction might have been underestimated. Second, the definition of TDF-related nephrotoxicity, especially the criteria used to evaluate proximal renal tubular damage, is not uniformly established in the field and is different in the published studies. Accordingly, we

decided to adopt changes in eGFR, instead of parameters for proximal renal tubular damage. Using the eGFR as a marker for TDF-associated renal dysfunction, our results might have underestimated the incidence of TDF-related renal tubular dysfunction. However, the result of this study could be informative to resource-limited settings, where it is difficult to evaluate renal tubular markers. The rationale and limitation of adopting more than 25% decrement in eGFR as the criterion for renal dysfunction were discussed in detail in our previous study [16]. Third, our cohort was characterized by the high prevalence of ritonavir-boosted PI use, which is considered by some groups a risk for TDF-related nephrotoxicity [24]. While it is difficult to completely exclude the impact of concurrent ritonavir-boosted PI in this study, it should be noted that the use of ritonavir-boosted PIs did not correlate with renal dysfunction in univariate analysis in this cohort (Table 2). Fourth, the study subjects were mainly men (mostly men who have sex with men and very few injection drug users). Further studies are needed to determine whether the findings of this study are also applicable to females, patients with different route of transmissions, and patients of different racial background.

In conclusion, the present study demonstrated a high incidence of renal dysfunction with TDF use, compared to ABC, among treatment-naïve patients with low body weight. TDF use was identified as an independent risk for renal dysfunction in a

statistical model that included TDF as a primary exposure. At 96 weeks, patients with TDF showed greater eGFR decrement than patients treated with ABC. TDF is certainly a drug of choice in the treatment of HIV infection, but the importance of close monitoring of renal function in patients with small body weight, especially those with baseline body weight <60 kg should be emphasized for early detection of TDF-related nephrotoxicity. Further studies are warranted to elucidate the long-term prognosis of renal function with TDF use in patients with low body weight.

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Author Contributions

Conceived and designed the experiments: TN HK HG TS EK JT SO. Performed the experiments: TN HK TS TA KW EK MH. Analyzed the data: TN HK HG TS HH HY K. Tsukada MH K. Teruya YK. Contributed reagents/materials/analysis tools: TA KW HH JT HY K. Tsukada MH K. Teruya YK. Wrote the paper: TN HK HG TS EK SO.

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The relationship between HIV testing and CD4 counts at HIV diagnosis among newly diagnosed HIV-1 patients in Japan

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Summary: The aim of this study was to investigate the factors relating to CD4 level at HIV diagnosis and HIV testing behaviour. Participants were newly diagnosed patients ($n = 654$) in Japan from 2000 to 2005. Around 75% of participants were diagnosed at hospital and clinics. Mean CD4 counts at diagnosis through voluntary HIV testing, screening tests and testing due to concomitant sexually transmitted infection (STI) were 368, 336 and 316 cells/ μL , respectively. In contrast, the mean CD4 count where testing was due to the presence of HIV-related clinical symptoms was 151 cells/ μL ($P < 0.0001$). Compared with those diagnosed at their first HIV test, those who had undertaken multiple HIV tests prior to diagnosis showed CD4 counts that increased significantly ($P < 0.0001$) in relation to the number of tests undertaken: CD4 count at first test was 232 cells/ μL , second test 346 cells/ μL and third or additional tests 439 cells/ μL . According to our results, HIV testing policy that promotes HIV testing in medical settings and among STI patients is needed to facilitate earlier HIV diagnosis in Japan.

Keywords: HIV, testing, early diagnosis, Japan, CD4 count

INTRODUCTION

Combination antiretroviral therapy has dramatically reduced the morbidity and mortality of HIV-1 infection and has led to the extension of long-term survival rates for people with HIV and AIDS.¹⁻³ Late diagnosis is currently the major factor that increases the risk of morbidity and mortality related to HIV infection. Short-term mortality (death within a year of diagnosis) has been reported to be 6.1% for patients diagnosed late (CD4 counts < 200 cells/ μL) and 9.7% for patients who start therapy with CD4 counts < 50 cells/ μL .^{4,5} An increase in the proportion of people with HIV who are aware of their serostatus can also contribute to the prevention of HIV infection transmission;⁶ thus earlier diagnosis has benefits for the patient as well as for public health promotion.

Incidence and factors relating to the late diagnosis of HIV-1 infection have been investigated in a number of countries,⁷⁻¹¹ and factors identified as contributing to late diagnosis include being male, heterosexual, older in age and from minority racial/ethnic groups. Greater access to HIV testing for those infected is necessary in order to reduce late diagnosis.¹² In 2001, the Centers for Disease Control and Prevention (CDC) in the USA proposed routine testing for high-risk groups, such as patients with sexually transmitted infections (STIs) or tuberculosis, men who have sex with men (MSM), injecting drug users and people with multiple sexual partners.¹³

Furthermore, in 2006, new CDC recommendations have promoted 'opt-out' screening for HIV-1 infection, in which medical professionals offer HIV testing as a part of routine clinical care for all patients aged 13-64 years in all health-care settings. Recommendations also state that health-care providers should facilitate screening for HIV-1 infection at least annually for all people likely to be in a high-risk category.¹⁴ Actually, the number of people who have undergone HIV testing has increased 3.4-6.8 times due to routine testing or opt-out screening.^{15,16} Opt-out screening in antenatal care has led to a reduction in undiagnosed maternal infection, and to higher CD4 counts at diagnosis in pregnant women than in non-pregnant women.¹⁷ Cost and consequence evaluation has found that opt-out screening may lead to increased diagnosis of patients with unknown HIV infection.¹⁸ However, due to insufficient investigation, it is not yet known whether opt-out screening is effective for earlier detection and improved prognosis of HIV-1 infection in general populations.

Japanese Ministry of Health surveillance data report 13,894 cases of HIV infection to the end of 2007, which translates to the cumulative number of HIV/AIDS patients per 100,000 population as 10.9 nationally, and 38.7 in Tokyo. The number of newly infected HIV/AIDS patients has been increasing annually and in 2007 the number of reported new HIV diagnoses was 1500 cases. Among new cases reported in 2007 approximately 30% had developed AIDS at the time of diagnosis. Incidence modelling indicates that the actual number of people with undiagnosed HIV is estimated to be 4.2 times higher than the reported number of cases.¹⁹ Given the large number of patients who had developed AIDS at the time of

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diagnosis and the high level of undiagnosed HIV-1 infection in Japan, it is a priority to identify factors associated with early and late HIV-1 diagnosis. Therefore, the aim of this study was to clarify the factors for early and late diagnosis by investigating when and why HIV patients undertake HIV testing, their CD4 counts at diagnosis, and the number of previous HIV tests undertaken prior to diagnosis.

METHODS

Study population

Patients with newly diagnosed HIV-1 infection who attended the AIDS Clinical Center at the National Center for Global Health and Medicine from April 2000 to March 2005 were included in this study. The AIDS Clinical Center is the largest national HIV treatment and research centre in Japan, treating approximately 40% of HIV patients in Tokyo and the surrounding regions.

The study was approved by the Ethics Committees of the National Center for Global Health and Medicine and the University of Tsukuba. Written consent from respondents was not required because the study was a retrospective observational study. The study protocol was publicized on the University Hospital Medical Information Network-Clinical Trials Registry database and followed the ethical guidelines for epidemiological studies set by the Japanese Ministry of Health, Labor and Welfare.

As the study aimed to analyse the risk behaviours of patients with HIV-1, patients with perinatally and occupationally acquired HIV were excluded. Study participation was limited to Japanese nationals diagnosed with HIV infection in Japan because the HIV testing experiences of non-Japanese people is affected by visa type, health insurance status, and accessibility to HIV information and testing services.

Data collection

Data collection was conducted from a medical record review of patient medical charts. Data were collected on patient demographics (age, gender, sexual orientation), CD4 count at diagnosis, stage of HIV-1 infection, place of HIV testing, reasons for undertaking the HIV test and the number of HIV tests undertaken before diagnosis. Patients with CD4 counts <200 cells/ μ L at diagnosis were defined as 'late testers' in this study. CD4 count at diagnosis was defined as the count closest to, and within six months of, the date of diagnosis. AIDS patients were defined according to the CDC category C disease listing,²⁰ regardless of CD4 count at diagnosis.

Reasons for HIV testing

Reasons for HIV testing were classified into four categories: (1) voluntary, (2) screening for other reasons, (3) concomitant STI-related testing and (4) clinical symptoms of HIV-1 infection. The voluntary group included patients who undertook the test voluntarily at any site (for example, public health centres, HIV testing centres, private clinics and self-conducted home testing). The screening group included patients who undertook tests before surgery, before blood donation, as part of a health check-up, and for antenatal screening. The STI group consisted of patients who were advised to undertake HIV testing by a medical provider following an STI diagnosis. Patients in the

fourth group had clinical signs or symptoms suggesting HIV-1 infection (for example, *Pneumocystis Pneumonia*, oropharyngeal or oesophageal candidiasis, aseptic meningitis, fever of unknown origin and lymphadenopathy) and were offered HIV testing by a medical provider on the basis of these signs or symptoms.

Statistical analyses

The mean CD4 counts at diagnosis were compared according to the reason for testing and number of tests undertaken, using one-way analysis of variance and a Tukey multiple comparison test. The characteristics of patients with CD4 counts <200 cells/ μ L at diagnosis were examined using the chi-square test. Logistic regression analysis was used to assess associations between CD4 counts <200 at diagnosis and patient characteristics. All statistical analyses were performed by using SPSS v.14.0 (SPSS Inc, Chicago, IL, USA). *P* values of <0.05 were considered statistically significant.

RESULTS

Patient characteristics

A total of 830 new patients visited the AIDS Clinical Center for consultation from April 2000 to March 2005. Among these, 110 foreigners, four patients with perinatal HIV transmission, one patient with occupational exposure and 16 patients diagnosed outside Japan were excluded from the study. Additionally, 45 patients were excluded because of insufficient data regarding CD4 count within the six months following HIV-1 diagnosis, sexual orientation, reason for HIV testing and/or number of tests taken previously. As a result, 654 patients were identified as being eligible for the study. Participant characteristics are shown in Table 1. The participant characteristics in this study

Table 1 Characteristics of newly diagnosed HIV-1 patients from April 2000 to March 2005 (*n* = 654)

Characteristics <i>n</i> = 654	<i>n</i>	%
Gender		
Men	603	92.2
Women	51	7.8
Age (years)		
19–29	199	30.4
30–39	245	37.5
40–49	102	15.6
\geq 50	108	16.5
Sexual orientation		
Homosexual/bisexual	538	82.3
Heterosexual	116	17.7
HIV stage		
AIDS	189	28.9
Non-AIDS	391	59.8
Primary infection	74	11.3
CD4 count at HIV-1 diagnosis		
0–49	144	22.0
50–199	132	20.2
200–349	156	23.9
350–499	122	18.6
\geq 500	100	15.3
Place of HIV testing for HIV diagnosis		
Hospital	425	65.0
Clinic	77	11.8
Public health centre/HIV testing site	130	19.9
Other*	22	3.4

*Other included 18 subjects for blood donation, three for mail-order test and one for prison intake