to that of HLA-A- or HLA-B-restricted CTL [28]. The present study also demonstrated that two HLA-C-restricted CTL had a strong ability to suppress HIV-1 replication *in vitro*. These findings suggested the possibility that some HLA-C-restricted T cells can control HIV-1 *in vivo*.

Previous studies demonstrated that HLA-A-restricted or HLA-B-restricted HIV-1-specific CTL recognised target cells infected with NL-432 M20A mutant (one amino acid substitution of Ala for Met at residue 20 of Nef), which lost the ability to down-regulate HLA-A and HLA-B molecules in HIV-1-infected cells, much more than those infected with NL-432 [12, 35, 39]. For example, Gag263-10-specific and Rev77-9-specific CTL showed approximately 50% suppression of the M20A virus replication but did not suppress NL432 replication (Supporting Information Table 1). Thus, HLA-C-restricted CTL, which is not affected by the Nef-mediated HLA down-regulation, have an advantage in the recognition of HLA–epitope complex on HIV-1-infected cells *in vivo*.

Previous population studies analysing HIV sequences in African cohorts demonstrated that some amino acid substitutions of HIV-1 Gag, Pol, and Nef are associated with HLA-C alleles [32, 33]. These studies suggested possibility that these substitutions are escape mutations selected by HLA-C-restricted T cells. However, since they did not demonstrate that specific CTL failed to recognise these substitutions, it still remained unclear whether HLA-C-restricted T cells could select escape mutant. We demonstrated here that the Pol463-10-specific CTL failed to kill the 9A mutant-infected cells but effectively killed WT HIV-1-infected ones. In addition, the CTL had a strong ability to suppress replication of a WT of HIV-1 but no ability to suppress that of the 9A mutant. The longitudinal analysis of HLA-Cw*1202$^+$ HIV-1-infected individuals showed the mutation from the WT to the 9A mutant. These results together support the idea that HLA-C-restricted CTL selected this escape mutant *in vivo*.

A previous study on a cohort infected with HIV-1 clade C virus demonstrated that HLA-C allele-associated Pol mutations are associated with low VL [33], suggesting these mutations increase fitness cost. The present study also demonstrated that NL-432 carrying the 9A mutant had a higher fitness cost than NL-432. However, the analysis of HLA-Cw*1202$^+$ individuals having and not having this mutation showed no association between VL and the presence of this mutation (data not shown). These suggest the possibility that a complementary substitution may compensate the effect of the 9A in terms of fitness cost. Another explanation is that fitness cost of the 9A mutant virus is not so much higher than that of the WT virus *in vivo*. Indeed, the difference in fitness cost between the two viruses in primary CD4$^+$ T cells is much smaller than that between them in the cell lines.

Both Pol 328-9-specific and Pol 463-10-specific CTL had strong ability to suppress HIV replication *in vitro*. However, the latter CTL selected escape mutants whereas the former CTL did not. It remains unknown why the one could select an escape mutant but the other could not. A recent study demonstrated that HLA-A*1101-restricted Nef73-specific and Nef84-specific CTL clones have strong ability to suppress HIV-1 replication *in vitro* but that the latter CTL can select an escape mutant whereas the former one did not [37]. *Ex vivo* analysis of these CTL showed that Nef84-specific CTL have a stronger ability to recognise the epitope than the Nef73-specific CTL [37]. That study suggested that only CTL having a strong ability to recognise the epitope can suppress HIV-1 replication *in vivo* so that escape mutants may be selected. This might be the case also for these HLA-Cw*1202-restricted CTL.

A variant 35 kb upstream of the *HLA-C* gene (*−35C/T*) was previously shown to be associated with the HLA-C mRNA expression level and steady-state plasma HIV RNA levels [29]. A recent study analysing 1698 European American individuals demonstrated that the *−35CC* allele is a proxy for high cell surface expression of HLA-C and that individuals with this allele progress more slowly to AIDS and control viremia significantly better than those without this low allele [40]. HLA-Cw*1202 is frequently found in east-Asia including Japan and forms a haplotype with HLA-A*2402 and HLA-B*5201. HLA-Cw*1202 is known to be highly associated with *−35CC* allele [40]. Therefore, we speculate that HLA-Cw*1202 is associated with a slow progression to AIDS.

In the present study, we demonstrated that HLA-Cw*1202-restricted Pol 463-10-specific CTL, which had a strong ability to suppress HIV-1 replication, selected an escape mutant, indicating that HLA-C allele-restricted HIV-specific CTL also play an important role in the generation of HIV-1 polymorphism. Further analysis of HLA-C-restricted CTL is expected to clarify the role of HLA-C alleles in HIV-1 infections.

Materials and methods

Samples of HIV-1-infected individuals

Plasma and PBMC were separated from whole blood of chronically HIV-1-infected individuals. The National Center for Global Health and Medicine and the Kumamoto University Ethical Committee approved this study. Informed consent was obtained from all subjects according to the Declaration of Helsinki.

HLA-typing

The HLA type of the chronically HIV-1-infected individuals was determined by standard sequence-based genotyping.

Synthetic peptides

We previously designed and generated overlapping peptides consisting of 11-mer or 17-mer amino acids in length and spanning Gag, Pol, and Nef of HIV-1 clade B consensus sequences [41, 42]. Each 11-mer and 17-mer peptide was overlapped by at least 9 and 11 amino acids, respectively.

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Sequence of autologous virus

Viral RNA was extracted from plasma samples from HIV-1-infected individuals using a QIAamp MinElute virus spin kit (QIAGEN). cDNA was synthesised from the viral RNA using Cloned AMV First-Strand cDNA Synthesis kit (Invitrogen). The Pol regions including the two epitopes was amplified by nested PCR, and amplified products were used for sequencing reaction by BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems). DNA sequencing was performed by ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

Cells

The EBV-transformed B-LCL were generated by transforming B cells from PBMC of healthy volunteers and HIV-1-seropositive individuals, KI-069, and KI-108. C1R cells expressing HLA-Cw*1202 (C1R-HLA-Cw*1202) were generated by transfecting C1R cells with the HLA-Cw*1202 gene; 721.221-CD4-HLA-Cw*1202 cells were generated by transfecting 721.221-CD4 cells with HLA-Cw*1202 genes. These transfectants were cultured in RPMI 1640 supplemented with 10% FBS and 0.15 /mL hygromycin B. H9 cells were cultured in RPMI 1640 supplemented with 10% FBS. MGIC-5 cells (CCR5-transduced HeLa-CD4/LTR-β-gal cells) were cultured in DMEM supplemented with 10% FBS as described previously [43].

Generation of 2 HLA-Cw*1202-restricted HIV-1-specific CTL clones

The two Pol-epitope-specific CTL clones were generated from bulk CTL specific for Pol328-9 or Pol463-10 epitopes as described previously [37].

Generation of NL−432−$_{Pol\ 463-10-9A}$ mutant clones

The NL-432-$_{Pol\ 463\text{-}10\text{-}9A}$ mutant virus was generated by introducing the Pol463-10-9A mutation into NL-432 using site-directed mutagenesis (Invitrogen).

Intracellular cytokine assay

PBMC from HLA-Cw*1202-positive HIV-1-infected patients were stimulated with HIV-1-derived peptide (1 μM) in culture medium (RPMI 1640 medium supplemented with 10% FBS and 200 U/mL recombinant human IL-2). After 14 days in culture, the cells were assessed for IFN-γ production using a FACS Calibur (BD Bioscience). Briefly, bulk cultures were stimulated with HLA-Cw*1202-expressing cells pulsed with HIV-1-derived peptide (1 μM) for 2 h at 37°C. Brefeldin A (10 μg/mL) was added, and incubated for a further 4 h. The cells were collected and stained with PE-labelled anti-CD8 mAb (Dako, Glostrup, Denmark). Cells were fixed with 4% paraformaldehyde solution, and permeabilised with permeabilization buffer (0.1% saponin and 20% Newborn Calf Serum in PBS) at 4°C for 10 min, followed by staining with FITC-labelled anti-IFN-γ mAb (PharMingen, San Diego, CA).

CTL assay for target cells pulsed with HIV-1 peptide

Cytotoxic activity of HIV-1-specific CTL was measured by the standard ^{51}Cr release assay, as previously described [12]. Briefly, target cells were labelled by $Na_2^{51}CrO_4$, then washed three times with RPMI 1640-10% FBS. ^{51}Cr-labelled target cells were plated 96-U plate with or without 1 μM peptide, and incubated for 1 h. After 1 h of incubation, CTL clones were added and incubated for 4 h. The supernatants were harvested and measured by a γ counter.

CTL assay for target cells infected with HIV-1

721.221-CD4-HLA-Cw*1202 cells were exposed to NL-432 or NL-432-$_{Pol\ 463\text{-}10\text{-}9A}$ for 3–6 days. Infection rate of these cells were measured by staining HIV-1 p24 Ag (KC57-FITC; Beckman Coulter). When approximately 30–60% of cells were infected, ^{51}Cr-labeled infected cells were co-cultured with CTL clones for 6 h. The supernatants were harvested and measured by a γ counter.

Replication suppression assay

The ability of HIV-1-specific CTL to suppress HIV-1 replication was examined as previously described [41]. Briefly, CD4$^+$ T cells were incubated with a given HIV-1 clone for 6 h at 37°C. After three washes with RPMI 1640-10% FBS, the cells were co-cultured with HIV-1-specific CTL clones. From day 3 to day 9 post infection, 10 μL of culture supernatant was collected; and the concentration of p24 Ag in it was measured with an enzyme immunoassay (HIV-1 p24 Ag ELISA kit; ZeptoMetrix, Buffalo, NY). The percentage of suppression of HIV-1 replication was calculated as follows: % suppression = (1–concentration of p24 Ag in the supernatant of HIV-1-infected CD4$^+$ T cells cultured with HIV-1-specific CTL/concentration of p24 Ag in the supernatant of HIV-1-infected CD4$^+$ T cells cultured without the CTL) × 100.

p24 production assay

H9 cells (8×10^5) and CD4$^+$ T cells (8×10^5) were exposed to each infectious virus preparation (500 blue cell-forming units in MAGIC-5 cells) for 6 h, washed twice with PBS, and cultured in 5 mL of complete medium [43]. The culture supernatants (0.2 mL) were harvested every other day, and the volume removed was replaced with fresh medium. The concentration of p24 Ag was measured with of an enzyme immunoassay (HIV-1 p24 Ag ELISA kit; ZeptoMetrix). Replication kinetics assays were performed in duplicate.

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Competitive HIV-1 replication assay

Freshly prepared H9 cells (3×10^5) and CD4$^+$ T cells (3×10^5) were exposed for 2 h to mixtures of paired virus preparations (various blue cell-forming units) for examination of their replication ability, washed twice with PBS, and cultured as described previously [43]. Every other day the supernatant was harvested, and then cDNA was synthesised and sequenced. The change in viral population was determined from the relative peak height on sequencing electrograms.

ELISPOT assay

ELISPOT assay was performed as previously described [37]. Briefly, cryopreserved PBMC of 25 HLA-Cw*1202$^+$ HIV-1-infected individuals were plated in 96-well polyvinylidene plates precoated with 0.5 μg/mL of anti-IFN-γ mAb 1-DIK (Matbech, Stockholm, Sweden). The appropriate amount of Pol 328-9 or Pol 463-10 peptide and PBMC were added at 1×10^5 cells/well and then the plates were incubated for 40 h. After the addition of biotinylated anti-IFN-γ mAb at 0.5 μg/mL, plates were incubated at room temperataure for 100 min. and then washed with PBS. Subsequently, streptavidin-conjugated alkaline phospatase was added, followed by 40 min incubation at room temperature. Individual cytokine-producing cells were detected as dark spots after a 20-min reaction with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium using an alkaline phosphatase-conjugate substrate (Bio-Rad, Richmond, CA).

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Abbreviations: **B-LCL:** B-lymphoblastoid cell lines · **V9A:** V-to-A substitution at the 9th position · **VL:** viral load

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Original article

Effective recognition of HIV-1-infected cells by HIV-1 integrase-specific HLA-B*4002-restricted T cells

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Abstract

HLA-B*4002 is one of the common HLA-B alleles in the world. All 7 reported HLA-B*4002-restricted HIV epitopes are derived from Gag, Nef, and Vpr. In the present study we sought to identify novel HLA-B*4002-restricted HIV epitopes by using overlapping 11-mer peptides of HIV-1 Nef, Gag, and Pol, and found that 6 of these 11-mer Pol peptides included HLA-B*4002-restricted epitopes. Analysis using truncated peptides of these 6 peptides defined 4 optimal Pol (integrase) epitopes. All epitopes previously reported had Glu at position 2 (P2), suggesting that Glu at P2 is the anchor residue for HLA-B*4002; whereas only 2 of the integrase epitopes that we here identified had Glu at P2. CTL clones specific for the 2 epitopes effectively recognized HIV-1-infected cells whereas those for other 2 epitopes only weakly recognized them. The antigen sensitivity of the former clones for the epitope peptide was much higher than that of the latter clones, suggesting 2 possibilities: 1) the former T cells have high-affinity TCRs and/or 2) the epitope peptides recognized by the former T cells are highly presented by HLA-B*4002 in HIV-1-infected cells. These integrase-specific T cells with high antigen sensitivity may contribute to the suppression of HIV-1 replication in HIV-1-infected HLA-B*4002$^+$ individuals.
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Keywords: HIV-1; Cytotoxic T lymphocytes; HLA-B*4002; Integrase

1. Introduction

Human immunodeficiency virus type 1 (HIV-1)-specific cytotoxic T lymphocytes (CTL) play an important role in HIV-1 infections [1–4]. Previous studies demonstrated that HIV-1-specific CTL can inhibit viral replication in vitro [5–7] and that depletion of CD8$^+$ T cells by treatment with an anti-CD8 mAb results in failure of the clearance of the virus in rhesus macaques infected with chimeric simian/human immunodeficiency virus [8]. These studies suggest that the CD8$^+$ CTLs contribute to viral clearance and disease progression in HIV-1-infected individuals. The study of CTL responses in an African cohort demonstrated that HLA-B-restricted T cell responses are associated with lower viral load than HLA-A-restricted or HLA-C-restricted ones [9], suggesting that HLA-B-restricted responses are important for the control of HIV-1. Therefore, the characterization of HIV-1 epitope-specific HLA-B-restricted CTLs is important for understanding the pathogenesis of HIV and developing an AIDS vaccine.

HLA-B*4001 and HLA-B*4002 are common HLA-B alleles in the world. These alleles are found in 10.8% and 16.6% of Japanese population, respectively, and the frequency of HLA-B*4002 is the third highest among HLA-B alleles [10]. Only residue 97 differs between these 2 alleles. So far 10 HLA-B*4001-restricted and 7 HLA-B*4002-restricted HIV epitopes have been reported in Caucasian cohorts [11–16]. These HLA-B*4002-restricted epitopes were derived from

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Gag, Nef, and Vpr; whereas the HLA-B*4001-restricted ones came from Gag, Nef, Pol, and Env.

In the present study, we sought to identify HLA-B*4001-restricted and HLA-B*4002-restricted HIV-1 epitopes in chronically HIV-1-infected Japanese cohorts by using 11-mer overlapping peptides derived from Pol, Gag, and Nef. We focused on these 3 proteins in the present study because these major proteins, which provide many CTL epitopes, are considered as vaccine targets. In addition, $CD8^+$ T cell clones specific for these newly identified epitopes were generated and used to clarify their ability to recognize HIV-1-infected cells. In the present study, we found 4 novel integrase epitopes presented by HLA-B*4002 and further characterized the $CD8^+$ T cells specific for these epitopes. Two of these epitopes were considered as immunodominant epitopes, because the specific T cells effectively recognized HIV-1-infected cells.

2. Materials and methods

2.1. Samples of HIV-1-infected individuals

This study was approved by the National Center for Global Health and Medicine and the Kumamoto University Ethical Committee. Informed consent was obtained from all subjects according to the Declaration of Helsinki. Peripheral blood mononuclear cells (PBMCs) were separated from heparinized whole blood. The HLA type of the patients was determined by standard sequence-based genotyping.

2.2. Synthetic peptides

We previously designed and generated overlapping peptides consisting of 11-mer amino acids and spanning Gag, Pol, and Nef of HIV-1 clade B consensus sequences [17]. Each 11-mer peptide was overlapped by 9 amino acids. Truncated peptides of some 11-mer peptides were synthesized by utilizing an automated multiple peptide synthesizer and purified by high-performance liquid chromatography (HPLC). The purity was examined by HPLC and mass spectrometry. Peptides with more than 90% purity were used in the present study.

2.3. Cells

The EBV-transformed B-lymphoblastoid cell lines (B-LCL) were established by transforming B cells from PBMC of KI-400. C1R cells expressing HLA-A*0207 (C1R-A*0207) and those expressing HLA-B*4002 (C1R-B*4002) were generated by transfecting C1R cells with the HLA-A*0207 and HLA-B*4002 genes, respectively. C1R-A*3101 cells were previously generated [18]. 721.221-CD4 cells expressing HLA-B*4002 (.221-CD4-B*4002), HLA-Cw*0102 (.221-CD4-Cw*0102), and HLA-Cw*0304(.221-CD4-Cw*0304) were generated by transfecting 721.221-CD4 cells with the HLA-B*4002, HLA-Cw*0102, and HLA-Cw*0304 genes, respectively, and maintained in RPMI 1640 medium supplemented with 10% FCS and 2.0 mg/ml hygromycin B.

2.4. Intracellular cytokine production (ICC) assay

PBMCs from chronically HIV-1-infected patient KI-400 were stimulated with HIV-1-derived peptide (1 μM) in culture medium (RPMI 1640 medium supplemented with 10% FCS and 200 U/ml recombinant human IL-2). After 14 days in culture, the cells were assessed for IFN-γ production activity by using a FACSCalibur. Briefly, bulk cultures were stimulated with stimulator cells pulsed with HIV-1-derived peptide (1 μM) for 2 h at 37 °C. Brefeldin A (10 μg/ml) was then added, and the cultures were continued for an additional 4 h. Cells were collected and stained with phycoerythrin (PE)-labelled anti-CD8 monoclonal antibody (mAb; Dako Corporation, Glostrup, Denmark). After having been treated with 4% paraformaldehyde solution, the cells were made permeable by incubation in permeabilization buffer (0.1% saponin and 20% NCS in phosphate-buffered saline) at 4 °C for 10 min and then stained with fluorescein isothiocyanate (FITC)-labeled anti-IFN-γ mAb (PharMingen, San Diego, CA). After a thorough washing with the permeabilization buffer, the cells were analyzed by using the FACSCalibur. Similarly IFN-γ production of established CTL clones was analyzed by use of this assay.

2.5. Generation of CTL clones

Peptide-specific CTL clones were generated from established peptide-specific bulk CTLs by seeding 0.8 cells/well into U-bottomed 96-well microtiter plates (Nunc, Roskilde, Denmark) together with 200 μl of cloning mixture (RPMI 1640 medium containing 10% FCS, 200 U/ml human recombinant interleukin-2, 5×10^5 irradiated allogeneic PBMCs from a healthy donor, and 1×10^5 irradiated C1R-B*4002 cells pulsed with a 1 μM concentration of the appropriate HIV-1-derived peptides. Wells positive for growth after about 2 weeks were examined for CTL activity by performing the ICC assay. All CTL clones were cultured in RPMI 1640 containing 10% FCS and 200 U/ml recombinant human interleukin-2. CTL clones were stimulated biweekly with irradiated target cells pulsed with the corresponding peptides.

2.6. HIV-1 clones

NL-432, which is an infectious proviral clone of HIV-1, was previously reported [7,19].

*2.7. HIV-1 infection of .221-CD4-B*4002 and .221-CD4 cells*

.221-CD4-B*4002 and 721.221-CD4 cells were exposed to NL-432 for several days. These infected cells were used as stimulator cells for ICC assays when approximately 60% of cells had been infected, which was confirmed by intracellular staining for HIV-1 p24 antigen.

3. Results

*3.1. Identification of 11-mer peptides recognized by HLA-B*4001-restricted and HLA-B*4002-restricted HIV-1-specific CD8⁺ T cells*

To identify novel HLA-B*4001-restricted CTL epitopes, we analyzed 5 HIV-seropositive HLA-B*4001$^+$ Japanese individuals by Elispot assays with cocktails of overlapping 11-mer peptides spanning Gag (p17^{Gag}, p24^{Gag}, p2p7p1p6^{Gag}), Pol (Protease, RT, integrase), and Nef. The overlapping 11-mer peptide cocktails that gave more than 200 spots per 10^6 cells were used to stimulate PBMC of each patient in order to identify the epitopes. After the PBMC had been cultured for 2 weeks, their IFN-γ production was analyzed by using the ICC assay. We found that 3 peptide cocktails induced IFN-γ production. Further analysis using 10 peptides in the peptide cocktails showed that three 11-mer peptides included HLA-B*4001-restricted epitopes but all of these peptides contained reported HLA-B*4001-restricted epitope sequences. Thus, we could not find any novel HLA-B*4001-restricted epitopes.

In order to identify CTL epitopes restricted by HLA-B*4002, we analyzed fresh CD8$^+$ T cells from patient KI-400 (A*0207/A*3101, B*4002/B*4601, Cw*0102/Cw*0304) by performing Elispot assays with the cocktails of the overlapping 11-mer peptides. More than 200 spots per 10^6 cells were observed with 7 out of 25 Gag cocktails, 11 out of 50 Pol cocktails, and 1 out of 10 Nef cocktails (data not shown). To find novel HLA-B*4002-restricted CTL epitopes, we focused on analyzing 5 peptide cocktails (Gag21-49, Pol781-809, Pol801-829, Pol901-929, and Pol921-949) that did not contain reported epitopes restricted by the 6 HLA-class I alleles this patient expressed. To determine which peptide in each cocktail induced the specific CD8$^+$ T cells, we stimulated PBMCs from KI-400 with these peptide cocktails and then cultured the cells for 2 weeks. The responsiveness of the cultured CD8$^+$ T cells toward ten 11-mer peptides in each peptide cocktail was measured by using the ICC assay. IFN-γ production was found in the bulk CD8$^+$ T cells stimulated with autologous B-LCLs pre-pulsed with 2 Gag (Gag31-41 and Gag33-43) and 6 Pol peptides (Pol799-809, Pol807-817, Pol909-919, Pol911-921, Pol919-929, and Pol921-931).

For determination of HLA restriction molecules of CD8$^+$ T cells specific for these 11-mer peptides, the responsiveness of the bulk CD8$^+$ T cells towards peptide-pulsed C1R cells expressing one of the HLA-A or -B alleles or .221 cells expressing one of the HLA-C alleles was measured by performing the ICC assay. HLA-B*4002-restricted responses were found in the bulk culture cells stimulated with the cells pre-pulsed with Pol799-809, Pol807-817, Pol909-919, Pol911-921, Pol919-929 or Pol921-931 (data not shown). These results indicate that these six 11-mer peptides included HLA-B*4002-restricted epitopes.

*3.2. Identification of HLA-B*4002-restricted optimal epitope peptides*

To determine the optimal epitopes for these 11-mer peptides, we stimulated bulk T cells with C1R-B*4002 cells pre-pulsed with truncated peptide of Pol799-809, Pol807-817, Pol909-919, Pol911-921, Pol919-929 or Pol921-931 at concentrations of 1000 nM and then measured the IFN-γ production of each bulk T cells was measured by conducting the ICC assay. Previous studies on HLA-B*4002-restricted epitopes suggested that Glu at position 2 is an anchor for HLA-B*4002 (11–16). Judging from the finding that Pol801-811 did not include HLA-B*4002-restricted epitopes, we speculated that 2E in Pol799-809 (IG11: IEAEVIPAETG) would be the anchor for HLA-B*4002 rather than 4E. We therefore generated 5 truncated peptides (IT10: IEAEVIPAET, IA8: IEAEVIPA, ET9: EAEVIPAET, AT8: AEVIPAET, and AG9: AEVIPAETG) of Pol799-809 and investigated whether CD8$^+$ T cells induced by Pol799-809 would recognize these peptides. The T cells recognized only IG11 and IT10 at 1000 nM (Fig. 1A), whereas they showed higher sensitivity to IT10 than to IG11 (Fig. 1B). These findings indicate that Pol799-808 (IT10) was the optimal epitope.

For Pol807-817 (EL11: ETGQETAYFLL), we generated 4 truncated peptides (TL10: TGQETAYFLL, GL9: GQETAYFLL, GL8: GQETAYFL, and QL8: QETAYFLL). CD8$^+$ T cells induced by the Pol807-817 peptide recognized EL11, TL10, GL9 and QL8, but not GL8 (Fig. 1A), indicating that L at position 11 was critical for the epitope. On the other hand, the T cells showed higher sensitivity to EL11 than to the other 3 peptides (Fig. 1C). These findings indicate that Pol807-817 (EL11) was the optimal epitope.

For Pol909-919 (YI11: YSAGERIVDII) and Pol911-921 (AT11: AGERIVDIIAT), we assumed 2 possibilities: 1) the two 11-mer peptides shared the same epitope, or 2) the two peptides included different epitopes. To clarify these possibilities, we analyzed Pol909-919 and Pol911-921 independently. For Pol909-919, we generated 5 truncated peptides (SI10: SAGERIVDII, SI9: SAGERIVDI, AI8: AGERIVDI, AI9: AGERIVDII, and GI8: GERIVDII). CD8$^+$ T cells induced by Pol909-919 peptide recognized YI11, SI10, AI9, and GI8, but not SI9 and AI8 (Fig. 1A), indicating that I at position 11 was critical for this epitope. On the other hand, they showed higher sensitivity to GI8 than to the other 3 peptides (Fig. 1D). These findings indicate that Pol909-919 (GI8) was the optimal epitope. Regarding Pol911-921 (AT11), we generated 4 truncated peptides (AI9: AGERIVDII, AI8: AGERIVDI, GI8: GERIVDII, and GA9: GERIVDIIA). CD8$^+$ T cells induced by Pol911-921 peptide recognized AT11, AI9, GI8 and GA9, but not AI8 (Fig. 1A), indicating I at position 11 to be critical for this epitope. They also showed higher sensitivity to GI8 than to the other 3 peptides (Fig. 1E), indicating that GI8 (Pol912-919) was the optimal epitope. Thus, these results confirmed that Pol909-919 and Pol911-921 included the same epitope.

For Pol919-929 (IQ11: IATDIQTKELQ) and Pol921-931 (TQ11: TDIQTKELQKQ), we assumed that these two 11-mer peptides shared the same epitope. Therefore, we analyzed Pol919-929 and Pol921-931 independently. Regarding Pol919-929 (IQ11: IATDIQTKELQ) we speculated that 10L would be the C-terminus of the epitope because no hydrophilic residue is found in the C-terminus of HLA class I-binding peptides.

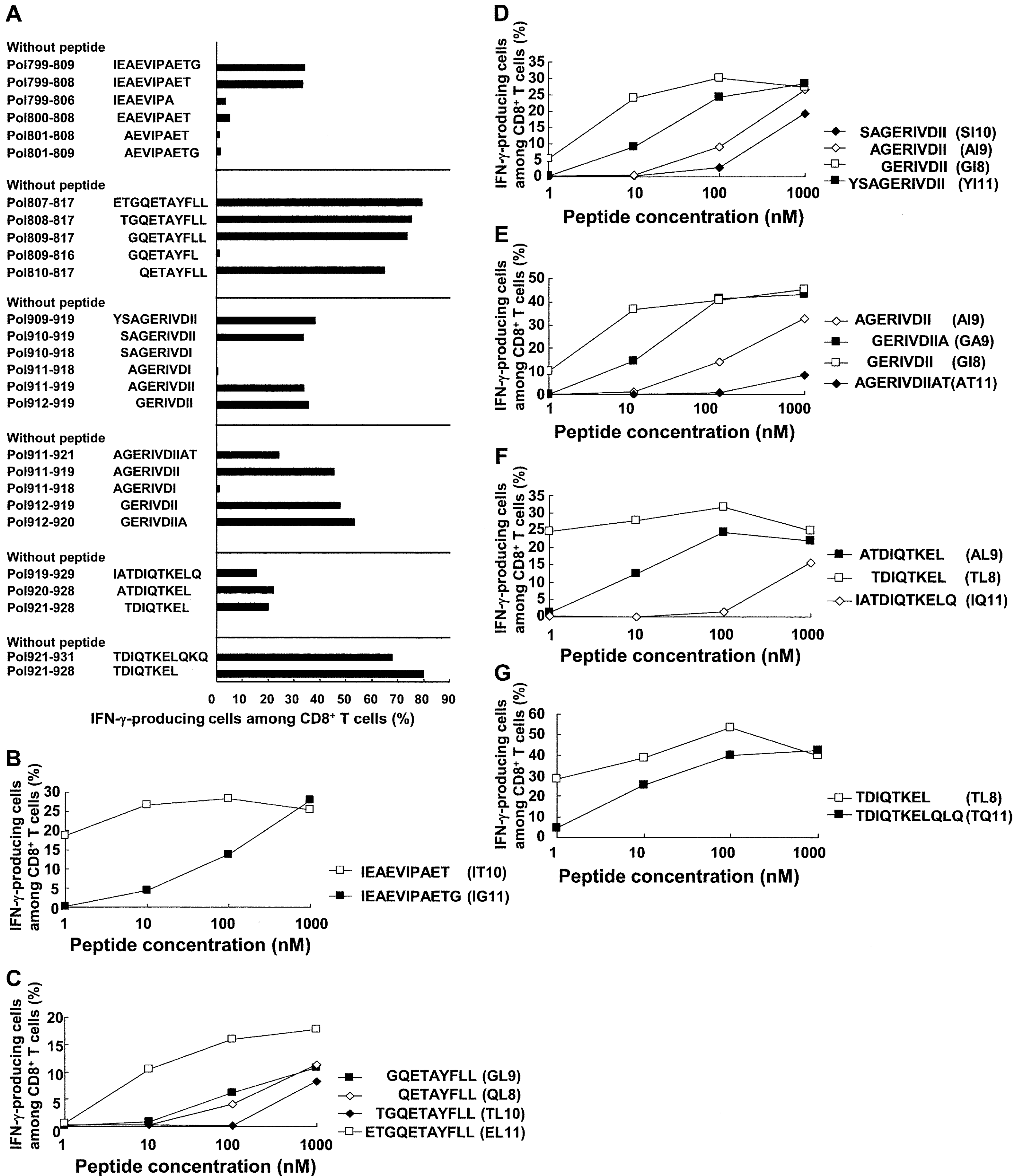

Fig. 1. Identification of HLA-B*4002-restricted HIV-1 CTL epitopes. A. For determination of the optimal epitopes of Pol799-809, Pol807-817, Pol909-919, Pol911-921, Pol919-929 and Pol921-931, the recognition of the bulk T cells for the truncated peptides was examined by using C1R-B*4002 cells pre-pulsed with each truncated peptide at a concentration of 1000 nM. The responsiveness of the bulk $CD8^+$ T cells toward each truncated peptide was measured by using the ICC assay. The percentages of IFN-γ-producing cells among the $CD8^+$ T cells are shown in the figure. B–G. Optimal epitopes were not determined at concentrations of 1000 nM for Pol799-809 (B), Pol807-817 (C), Pol909-919 (D), Pol911-921 (E), Pol919-929 (F) or Pol921-931 (G). The responsiveness of the bulk $CD8^+$ T cells was examined for C1R-B*4002 cells pre-pulsed with each truncated peptide at concentrations from 1 to 1000 nM. The responsiveness of the bulk $CD8^+$ T cells toward each truncated peptide was measured by performing the ICC assay. The percentages of IFN-γ-producing cells among $CD8^+$ T cells are shown in the figure.

Therefore, we generated 2 truncated peptides (AL9: ATDIQTKEL and TL8: TDIQTKEL). Bulk $CD8^+$ T cells induced by Pol919-929 peptide recognized all 3 peptides (Fig. 1A) and showed higher sensitivity to TL8 than to the other 2 peptides (Fig. 1F), indicating that Pol921-928 (TL8) was the optimal epitope. Similarly we speculated TL8 to be optimal epitope for Pol921-931 (TQ11: TDIQTKELQKQ), because no hydrophilic residue is found in the C-terminus of HLA-class I-restricted epitopes. Although bulk $CD8^+$ T cells induced by Pol921-931 peptide recognized both TQ11 and TL8 peptides (Fig. 1A), they showed higher sensitivity to TL8 than to TQ11 (Fig. 1F). These findings indicate that Pol919-929 and Pol921-931 11-mer peptides included the same epitope, Pol921-928(TL8).

Thus, we identified 4 HLA-B*4002-restricted optimal peptides. Interestingly, these 4 Pol epitopes were all derived from integrase.

3.3. Generation and antigen sensitivity of HLA-B*4002-restricted Pol-specific CTL clones

To analyze the $CD8^+$ T cells specific for these 4 integrase epitopes, IT10 (Pol799-808), EL11 (Pol807-817), GI8 (Pol912-919), and TL8 (Pol921-928), we established the specific $CD8^+$ T cell clones and analyzed them for their antigen sensitivity by using the ICC assays. The result was shown in Fig. 2. The T cell clones and their EC_{50} values were as follows: Pol799-808-specific T cells (27.7), Pol807-817-specific T cells (191.7), Pol912-919-specific T cells (443.1), and Pol921-928-specific T cells (7.6). These results indicate that Pol799-808-specific and Pol921-928-specific $CD8^+$ T cell clones had higher antigen sensitivity than Pol807-817-specific and Pol912-919-specific ones.

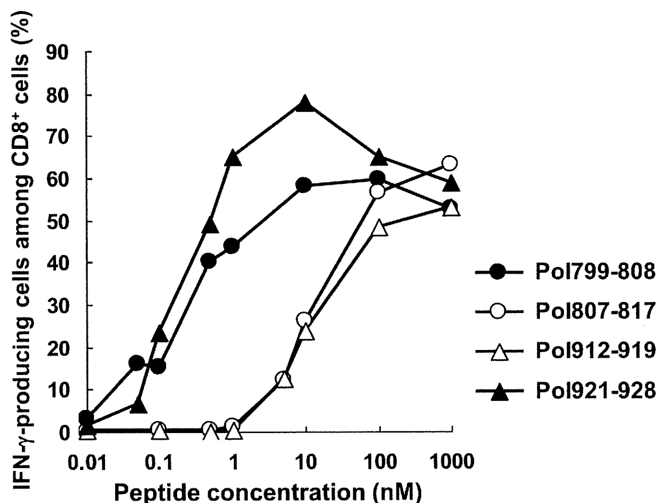

Fig. 2. Antigen Sensitivity of 4 HIV-1 integrase-specific $CD8^+$ T cells. Antigen sensitivity of 4 HIV-1 integrase-specific $CD8^+$ T cells was examined by using the ICC assay. The responsiveness of these CTL clones was examined for C1R-B*4002 cells pre-pulsed with each truncated peptide at concentrations from 0.01 to 1000 nM.

3.4. Recognition of HIV-1-infected cells by specific T cells

To clarify whether Pol799-808, Pol807-817, Pol912-919, and Pol921-928 were naturally occurring peptides and whether CTLs specific for these epitopes had the ability to recognize HIV-1-infected cells, we investigated the response of these peptide-specific $CD8^+$ T cell clones toward HIV-1 (NL-432)-infected .221-CD4 cell lines expressing HLA-B*4002. NL-432 includes wild-type sequences of these 4 epitopes. .221-CD4 cell lines and those expressing HLA-B*4002 were infected with NL-432, and then cultured for 4 days. The responses of the T cell clones toward these infected cells were measured by using the ICC assay. The percentage of the HIV-1-infected cells was determined by staining intracellular HIV-1 p24 (Fig. 3A). The Pol799-808-specific, Pol807-817-specific, Pol912-919-specific, and Pol921-928-specific CTL clones responded to .221-CD4-B*4002 cells infected with HIV-1 but not to uninfected .221-CD4-B*4002 cells or to HLA-B*4002-negative .221-CD4 cells infected with HIV-1. These results indicate that Pol799-808, Pol807-817, and Pol921-928 peptides were naturally processed and presented by HLA-B*4002 and that the T cells specific for these epitopes could recognize HIV-1-infected cells (Fig. 3B). On the other hand, the responses of Pol807-817-specific and Pol912-919-specific CTL clones was much weaker than those of the other CTL clones (Fig. 3B), indicating that the former CTLs only weakly recognized HIV-1-infected cells.

4. Discussion

There is only 1 amino acid substitution, at residue 97, on the peptide binding floor between HLA-B*4001 and HLA-B*4002. A previous study on the peptide motif of HLA-B*4001 showed that HLA-B*4001-binding peptide anchors are Glu at P2 (2E) and Leu at the C-terminus [20]. Indeed, 7 of 8 reported HLA-B*4001-restricted HIV-1-specific T cell epitopes have 2E and Leu at their C-terminus [11–13]. Although no HLA-B*4002-binding peptide motif had not yet been identified, we speculated that this motif would be similar to the HLA-B*4001-binding one. Indeed, all 7 HLA-B*4002-restricted epitopes previously reported have 2E (Table 1). However, 2 of the 4 epitopes identified in the present study did not have the 2E anchor. In addition, only 5 of 11 HLA-B*4002-restricted epitopes had Leu at their C-terminus. These findings suggest that the substitution from Ser to Arg at residue 97 may partially affect the structure of the F and B pockets. Pol807-817 (ETGQETAYFLL) does not have the 2E anchor. QL8 (QETAYFLL) is speculated to be an HLA-B*4002-restricted epitope because this peptide has 2E. However, the antigen sensitivity of the T cells specific for QL8 is much weaker than that for EL11. This result excludes the possibility that QL8 is the epitope peptide. Thr at position 2 of Pol807-817 may bind to the residues facing the B-pocket by hydrogen-bonding. Nine of the 11 HLA-B*4002-restricted epitopes have 2E, suggesting that the 2E is still anchor residue for HLA-B*4002.

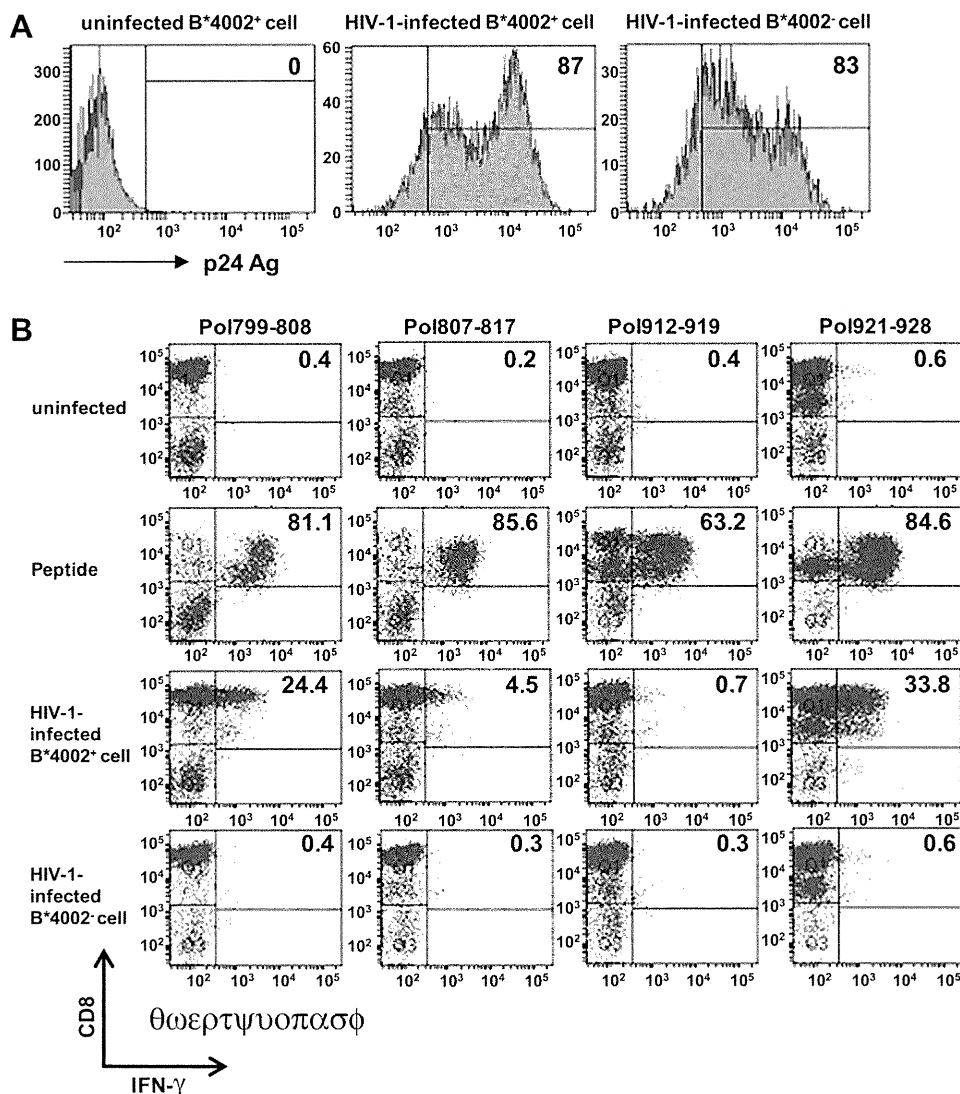

Fig. 3. Ability of 4 HIV-1 integrase-specific CD8$^+$ T cells to recognize HIV-1-infected cells. A. The .221-CD4 and B*4002$^+$.221-CD4 cell lines were infected with HIV-1 (NL-432) and cultured for 4 days. The frequency of HIV-1-infected cells was detected by using staining of intracellular p24 with anti-p24 mAb. The percentage of HIV-1-infected cells is shown in each figure. B. Recognition of HIV-1-infected cells by the Pol799-808-, Pol807-817-, Pol912-919- or Pol921-928-specific CD8$^+$ T cell clones. The activities of these peptide-specific CD8$^+$ T cell clones to recognize B*4002$^+$.221-CD4 cell lines infected with HIV-1 or those pre-pulsed with the corresponding peptide (1000 nM) were measured by use of the ICC assay. The percentages of IFN-γ-producing cells among CD8$^+$ T cells are shown in each figure.

Although the 7 HLA-B*4002-restricted epitopes previously reported do not include Pol-derived ones, we identified novel 4 HLA-B*4002-retricted Pol-specific T cell epitopes in the present study. Interestingly, all of these Pol epitopes were derived from integrase. Though 29 integrase epitopes were reported as 20 different HLA class I-restricted epitopes (Los Alamos HIV Molecular Immunology Data), integrase epitopes were not found among HLA-B*4001-restricted Pol epitopes. Regarding the integrase epitopes, HLA-B*4201 and HLA-B*1503 present 3 different epitopes, whereas the other 18 alleles present 1 or 2 epitopes. Thus, HLA-B*4002 is so far the only HLA-class I allele that can present more than 3 integrase epitopes.

Pol799-808-specific and Pol921-928-specific T cells strongly recognized HIV-1-infected cells, whereas Pol807-817-specific and Pol912-919-specific ones weakly recognized these cells. Antigen sensitivity of the former T cells was much higher than that of the latter ones. Thus, the ability to recognize HIV-1-infected cells was associated with the antigen sensitivity. However, it is difficult to clarify why the 2 T cells weakly recognize HIV-1-infected cells because we did not measure the bindings of these epitope peptides to HLA-B*4002 molecules and of the specific tetramers to the specific T cells. We can suggest 2 possibilities from the data shown in Fig. 2 and Fig. 3: 1) The former T cells may have higher affinity TCR and/or 2) these former epitope peptides are more highly presented than the latter by HLA-B*4002 in HIV-1-infected cells. Since Pol799-808-specific and Pol921-928-specific T cells strongly recognized HIV-1-infected cells, we proposed that they would effectively recognize and kill HIV-1-infected cells *in vivo*.

HLA-B*4001 and HLA-B*4002 are found in 10.8% and 16.6% of the Japanese population, respectively. Since both

Table 1
A list of HLA-B*4002-restricted epitopes identified previously and in this study.

Sequence	Protein	Reference
GELDRWEKI	Gag (p17)	*15
KETINEEAA	Gag (p24)	*15
AEWDRVHPV	Gag (p24)	*15
AEAMSQVTNS	Gag (p2p7p1p6)	*16
TERQANFL	Gag (p2p7p1p6)	*15
REPHNEWTL	Vpr	*14
KEKGGLEGL	Nef	*15
IEAEVIPAET	Pol (Integrase)	This study (Pol799-808)
ETGQETAYFLL	Pol (Integrase)	This study (Pol807-817)
GERIVDII	Pol (Integrase)	This study (Pol912-919)
TDIQTKEL	Pol (Integrase)	This study (Pol921-928)

HLA-class I alleles are detected in approximately 25% of Japanese individuals, T cell epitopes presented by these alleles are useful for studies on HIV-1 immunopathogenesis and the development of AIDS vaccines.

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Amebiasis in HIV-1-Infected Japanese Men: Clinical Features and Response to Therapy

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Abstract

Invasive amebic diseases caused by *Entamoeba histolytica* are increasing among men who have sex with men and co-infection of ameba and HIV-1 is an emerging problem in developed East Asian countries. To characterize the clinical and epidemiological features of invasive amebiasis in HIV-1 patients, the medical records of 170 co-infected cases were analyzed retrospectively, and *E. histolytica* genotype was assayed in 14 cases. In this series of HIV-1-infected patients, clinical presentation of invasive amebiasis was similar to that described in the normal host. High fever, leukocytosis and high CRP were associated with extraluminal amebic diseases. Two cases died from amebic colitis (resulting in intestinal perforation in one and gastrointestinal bleeding in one), and three cases died from causes unrelated to amebiasis. Treatment with metronidazole or tinidazole was successful in the other 165 cases. Luminal treatment was provided to 83 patients following metronidazole or tinidazole treatment. However, amebiasis recurred in 6 of these, a frequency similar to that seen in patients who did not receive luminal treatment. Recurrence was more frequent in HCV-antibody positive individuals and those who acquired syphilis during the follow-up period. Various genotypes of *E. histolytica* were identified in 14 patients but there was no correlation between genotype and clinical features. The outcome of metronidazole and tinidazole treatment of uncomplicated amebiasis was excellent even in HIV-1-infected individuals. Luminal treatment following metronidazole or tinidazole treatment does not reduce recurrence of amebiasis in high risk populations probably due to amebic re-infection.

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Introduction

Invasive amebiasis (IA) caused by *Entamoeba histolytica* is the second most common cause of mortality associated with parasitic infections worldwide, accounting for 40,000 to 100,000 deaths annually [1]. Amebiasis is transmitted by ingestion of food or water containing the cyst form of *E. histolytica*, which is prevalent in developing countries in Central and South America, Asia, and Africa. In the developed countries, most cases arise in travelers and immigrants from such endemic areas [2]. Recently, however, three developed East Asian countries (Japan, Taiwan, and South Korea) reported increased risk for amebiasis among men who have sex with men (MSM) due to oral-anal sexual contact [3–12]. The annual incidence of human immunodeficiency virus type 1 (HIV-1) infection is also increasing among MSM in these countries [13-17], resulting in growing concern on IA in HIV-1-infected MSM [6,9–12,18]. The recommended treatment for IA is metronidazole (750 mg t. i. d. for 10 days) or tinidazole (2 g q. d. for 3 days), followed by a luminal agent (paromomycin 500 mg t. i. d. for 10 days or diloxanide furoate 500 mg t. i. d. for 10 days) to eliminate intestinal colonization [18,19]. A previous report described no difference in the response to metronidazole or tinidazole treatment between HIV-1-positive and –negative IA patients [20]. However, the efficacy of luminal treatment in preventing recurrence, which can arise by relapse or re-infection, has not yet been assessed rigorously. In this study, we retrospectively analyzed 170 HIV-1-infected Japanese patients with IA, together with genomic typing of *E. histolytica* in 14 of these patients, and delineated the clinical features of IA in HIV-1-infected individuals and the efficacy of metronidazole, tinidazole and luminal treatment.

Methods

Ethics statement

The Institutional Review Board of National Center for Global Health and Medicine (Tokyo, Japan) approved this study. All patients who provided clinical samples for genotyping of *E. histolytica* gave written informed consent.

Case review

The medical records of HIV-1-infected cases diagnosed with IA at the AIDS Clinical Center, National Center for Global Health and Medicine, between April 1997 and March 2010, were reviewed. The diagnosis of IA was made when one of the following criteria was satisfied; 1) identification of and/or positive PCR (methods; see below) in clinical specimens (stool or punctuate-exudate) for erythrophagocytic trophozoites in patients with IA-

Author Summary

Amebiasis is usually transmitted by ingestion of contaminated food or water in developing countries. Recently, however, increased risk for amebiasis among men who have sex with men (MSM) due to oral-anal sexual contact was reported in developed countries, resulting in growing concern on amebiasis in HIV-1-infected MSM. The recommended treatment of amebiasis is metronidazole or tinidazole, followed by a luminal agent to eliminate intestinal cyst colonization. However, the efficacy of luminal treatment in preventing recurrence has not been assessed yet. In this study, we analyzed the medical records of 170 patients with amebiasis and HIV-1 co-infection. Treatment with metronidazole or tinidazole was excellent whereas luminal treatment did not reduce the frequency of recurrence of amebiasis. Recurrence was more frequent in those MSM with signs of sexual activity such as syphilis infection. Luminal treatment following metronidazole or tinidazole treatment does not reduce recurrence of amebiasis in high risk populations.

related symptoms, e.g., fever and liver abscess, or tenesmus and diarrhea, 2) high serum titer (>1:100) for antibody against *E. histolytica* in patients with IA-related symptoms in whom microbiological cultures or histological examination of clinical specimens did not identify any pathogen, and who showed improvement of IA symptoms following metronidazole or tinidazole monotherapy [10–12]. The medical records were surveyed for patients' characteristics, presenting forms of clinical IA [e.g., colitis, amebic liver abscess (ALA), and perianal abscess], HIV-1-induced immunocompromised status, and symptoms, laboratory data and serological markers of other sexually-transmitted diseases (STD) including syphilis, hepatitis B and C viruses (HBV and HCV). After completion of treatment for IA, the medical records were followed-up until March 2010, excluding those cases found to have died or lost to follow-up.

Genotyping of *E. histolytica*

To determine the strains of *E. histolytica* among HIV-1-infected Japanese patients, genotyping of *E. histolytica* was performed in patients who were PCR positive. The PCR method was used for the first time in our clinic for the diagnosis of amebiasis in December 2008, and since then 14 patients had been diagnosed as IA based on a positive PCR. For the PCR, DNAs were extracted from various biological specimens (e.g., stool, colon wash and punctuate-exudate) by using QIAamp DNA stool Mini Kit (Qiagen, Valencia, CA). Polymerase chain reactions were performed with specific sets of primers designed to target each of 6 loci (D-A, S-Q, R-R, A-L, S^{TGA}-D, and N-K) of tRNA-linked polymorphic short tandem repeats (STR), as described previously [21]. The PCR product was sequenced by ABI 3130XL Genetic Analyzer (Applied Biosystem, Foster city, CA) in both forward and reverse directions. Phylogenetic analysis and genotyping were performed as described previously [22].

Statistical analysis

Differences in patients' characteristics and clinical features were examined using the chi-square test or nonparametric test. The cumulative risk for recurrence was analyzed by the Kaplan-Meier method, and differences were tested by the log-rank test. The Cox proportional hazards model was used to assess the impact of luminal treatment on the recurrence rate after adjustment for other factors. The hazard ratio and 95% confidence interval were calculated. P values less than 0.05 were considered to denote statistical significance. All statistical analyses were performed using the Statistical Package for Social Sciences (SPSS Inc., Chicago, IL).

Results

Clinical data and response to treatment

IA was diagnosed in 170 HIV-1-infected cases between April 1997 and March 2010 (including amebic colitis, n = 102; ALA, n = 63; and perianal abscess, n = 5, Table 1). Thirty-three patients had two of the above three clinical forms of IA. All patients were males and 164/170 (96.5%) were MSM. High rates of positive TPHA (*Treponema pallidum* hemagglutination assay) (71.2%) and HBV exposure (HBs antigen-positive, HBs antibody-positive, or HBc antibody-positive) (60.0%) were observed. No significant differences were seen in CD4 counts, HIV-1 loads, coexisting AIDS definite disease and the proportion of patients treated with antiretrovirals, suggesting that HIV-induced immunocompromised status did not have an impact on the clinical presentation of amebic infection, in agreement with previous data [12]. In cases of amebic colitis (n = 102), diarrhea (69.7%) was the most common symptom followed by dysentery (55.9%) (Table 2). Fever (>37.5°C) was seen in only 20 patients (19.6%), including 5 cases with perforative peritonitis. In cases with ALA (n = 63), fever (95.2%) was the most common symptom followed by abdominal pain (55.6%). Diarrhea (46.0%) and dysentery (19.0%) were only seen in less than half of ALA cases. Single abscess (72.6%) was identified in most cases. Liver abscesses were seen more frequently in the right lobe (70.5%) than the left (9.8%). Nine patients (14.3%) had pleuritis (considered a co-existing disease), as well as abscesses in the right lobe, and 7 of these presented chest pain. Comparison of physical and laboratory data showed higher peak body temperature (BT), leukocyte count and C reactive protein (CRP) in ALA cases (Table 2) and perforative peritonitis cases (data not shown) compared with colitis cases, indicating that high fever, leukocytosis and high CRP could be the signs of extraluminal amebiasis. It is reported that high fever and leukocytosis are also common in ALA patients free of HIV-1 infection, though both parameters were unusually associated with simple amebic colitis [23]. In ALA cases, however, leukocyte count correlated positively with CD4 count (data not shown in tables: Pearson product-moment correlation coefficient 0.36, p value 0.004) and negatively with HIV-RNA load (Pearson product-moment correlation coefficient -0.28, p value 0.03), but CRP correlated neither with CD4 count nor HIV-RNA load (CRP-CD4, $p = 0.81$, CRP-HIV-RNA, $p = 0.32$). There were also no correlations between CD4 count, HIV-RNA load, BT, leukocyte count or CRP and abscess size or number.

All patients were treated with metronidazole (750 mg t. i. d. for 10 days) for IA, with the exception of two who were treated with tinidazole (2 g q. d. for 3 days). Complete remission of all IA symptoms was observed in 165 patients including the two treated with tinidazole. Five cases died within six months after diagnosis of IA; two from complications related to amebic colitis (one peritoneal perforation and one gastrointestinal bleeding), one from malignant lymphoma, one from *Pneumocystis jirovecii* pneumonia, and one from pulmonary thrombosis. The overall mortality rate was 3% in this study, which was comparable to those reported in non-HIV cases [2,23].

Recurrence after treatment

Luminal agents; paromomycin and diloxanide, are not approved in Japan, and they were not always available in our facility during the study period. After completion of IA treatment with metronidazole or tinidazole, luminal agents were administered when available. Consequently, 83 cases were treated with luminal

Table 1. Patient demographics, state of HIV, and serological markers.

	Colitis (n = 102)[1]	ALA (n = 63)[2]	Perianal abscess (n = 5)[3]	All (n = 170)	*P* value[4]
Age (years) [IQR]	38 [32–43]	37 [31–44]	45	38 [31–44]	0.58
Male sex (%)	102 (100)	63 (100)	5 (100)	170 (100)	–
Homosexual (%)	96 (94.1)	63 (100)	5 (100)	164 (96.5)	0.053
Past History of amebiasis (%)	16 (15.7)	9 (14.3)	1 (20.0)	26 (15.3)	0.81
CD4 count (/μl)	262 [98–398]	271 [123–411]	58	269 [107–403]	0.84
HIV-RNA (log copies/ml)	4.60 [3.89–5.32]	4.66 [3.91–5.11]	5.04	4.66 [3.93–5.28]	0.70
AIDS (%)	18 (17.6)	8 (12.7)	2 (40.0)	28 (16.5)	0.40
ART initiated (%)	18 (17.6)	11 (17.5)	1 (20.0)	30 (17.6)	0.98
TPHA test positive (%)	77 (75.5)	40 (63.5)	4 (80.0)	121 (71.2)	0.10
HBV exposure (%)	59 (57.8)	41 (65.1)	2 (40.0)	102 (60.0)	0.36
HCV Antibody positive (%)	3 (2.9)	3 (4.8)	0 (0)	6 (3.5)	0.42

Data are median [interquartile range: IQR] or number (percentage) of patients.
[1]5 cases of perforative peritonitis are included as co-existing diseases. Four cases were diagnosed coincidentally by colonoscopy in asymptomatic patients.
[2]31 cases of colitis, 1 case of perianal abscess, 9 cases of pleuritis, and 2 cases of peritonitis are included as co-existing diseases.
[3]1 case of colitis is included as co-existing diseases.
[4]Chi-square test or non-parametric test was performed for data of colitis and ALA.
UD: undetectable, ART: anti-retroviral therapy, TPHA test: *Treponema pallidum* Hemagglutination Assay test, HBV exposure: HBsAg-positive or HBsAb-positive, and/or HBc-Ab positive.
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agents; 38 cases with promomycin (500 mg t. i. d. for 10 days) and 45 cases with diloxanide furoate (500 mg t. i. d. for 10 days). No significant differences were seen in patients' characteristics, including HIV-1-induced immunocompromised status, serological markers of other STD, and clinical forms and severity of amebiasis between the 83 cases with luminal treatment and 82 cases who did not receive such treatment (Table S1). The median follow-up period after completion of metronidazole or tinidazole treatment was 50 months (inter quartile range: 19–85) in those who received luminal treatment, and 43 months (inter quartile range: 23–98) in those without.

Table 2. Clinical features of amoebic colitis and ALA.

	Colitis (n = 102)	ALA (n = 63)	*P* value
Symptoms			
Diarrhea (%)	71/102 (69.6)	29/63 (46.0)	0.003
Dysentery (%)	57/102 (55.9)	12/63 (19.0)	<0.001
Abdominal pain (%)	23/102 (22.5)	35/63 (55.6)	<0.001
Chest pain (%)	0/102 (0.0)	7/63 (11.1)	<0.001
Peak BT (°C) [IQR][3]	36.8 [36.5–37.4]	39.0 [38.8–39.5]	<0.001
WBC (/μ l) [IQR][3]	5,830 [4490–7580]	11,760 [9460–15170]	<0.001
CRP (mg/dl) [IQR][3]	0.62 [0.16–3.02]	19.15 [10.53–24.75]	<0.001
Frequency of diarrhea[1]			
≤ 5 times/day (%)	63/101 (62.4)	–	
6–10 times (%)	26/101 (25.7)	–	
≥ 11 times (%)	12/101 (11.9)	–	
Size of abscess (mm)	–	59 (10–180)	
Location of abscess[2]			
Right lobe only	–	43/61 (70.5)	
Left lobe only	–	6/61 (9.8)	
Both lobes	–	12/61 (19.7)	
Number of abscesses[1]			
Single (%)	–	45/62 (72.6)	
Multiple (%)	–	17/62 (27.4)	

[1]Data of one case were not available.
[2]Data of two cases were not available.
[3]Data are median [interquartile range: IQR] or number (percentage) of patients.
BT: body temperature, WBC: White Blood Cell counts, CRP: C reactive protein.
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Within the 12-month post-metronidazole treatment period, recurrence of IA was noted in only two patients who did not receive luminal treatment, suggesting reactivation of residual cysts of *E. histolytica* (Figure 1). However, during the entire follow-up period, six in each group experienced recurrence of IA, with no significant difference in the recurrence frequency by the log-rank chi-square test. Multivariate analysis showed that recurrence did not correlate with past history of IA, CD4 count, TPHA, HBV exposure (HBs antigen-positive or HBs antibody-positive), or the presence of extraluminal IA disease (Table 3). However, a positive HCV antibody was significantly associated with IA recurrence. Recurrence also tended to occur in those who acquired new syphilis infection during the follow-up period, though the difference did not reach statistical significance.

Genotypes of *E. histolytica*

Genotyping of *E. histolytica* was performed in samples obtained from 14 patients between December 2009 and March 2010 (colitis, n = 8; ALA, n = 4; colitis and ALA, n = 1; and perianal abscess, n = 1; Table S2). Eleven different genotypes were recognized, including five genotypes (J8, J12, J13, J20, and J23) identified previously in Japan [22], and six newly recognized genotypes (J24-J29). There was no significant relation between *E. histolytica* genotype and clinical presentation.

Discussion

In the present study, retrospective analysis of the medical records of 170 patients with HIV-1-infection and IA showed no

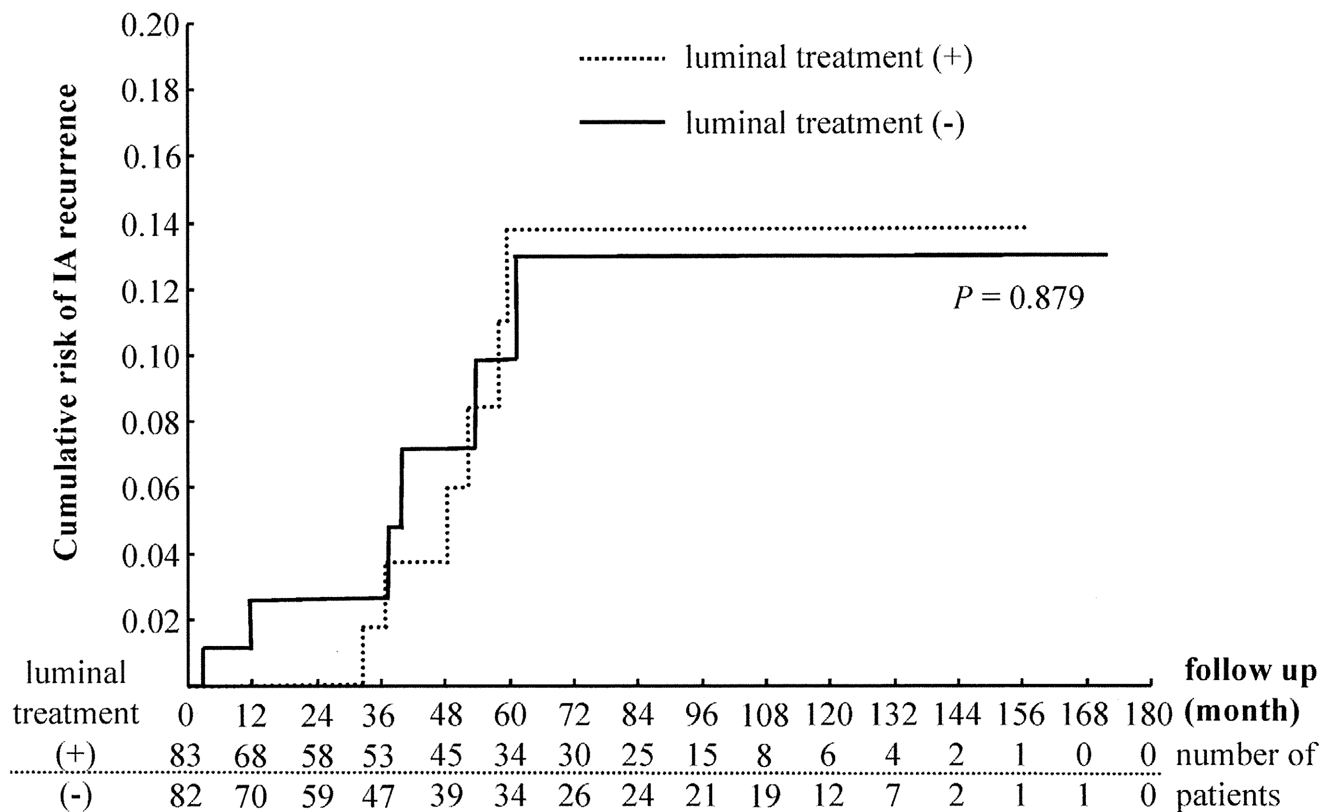

Figure 1. Kaplan-Meier estimates of time to IA recurrence. Cumulative probability of IA recurrence after completion of metronidazole or tinidazole treatment with or without subsequent luminal treatment.
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impact for HIV-1-induced immunocompromised status on the clinical forms of amebiasis. The physical and laboratory findings showed that high fever, leukocytosis and high CRP correlated with extraluminal diseases of amebiasis. In ALA cases, however, leukocyte count correlated positively with CD4 count and negatively with HIV-RNA load, indicating that CRP is more sensitive marker for the detection of the extraluminal diseases in advanced immunocompromised patients.

Only five patients died after the diagnosis of IA; two from IA complications and three from other causes. The results indicate excellent outcome for HIV-1-infected individuals with uncomplicated amebiasis treated with metronidazole or tinidazole, in agreement with previous reports on HIV and non-HIV cases [2,11,12,20,23]. Based on conventional wisdom and written opinion, adequate management of IA should include treatment with a luminal agent following metronidazole or tinidazole treatment, in order to eradicate residual cysts of *E. histolytica* due to the high rate (40–60%) of luminal colonization [2,23–27]. On the other hand, the results of longitudinal observational studies indicated that asymptomatic cyst carriers rarely develop IA, and

Table 3. Multivariate analyses for factors associated with frequency of recurrence.

	No recurrence (n = 153)[1]	Recurrence (n = 12)	Hazard ratio (95.0% CI)	*P* value
Past history of IA[2] (%)	24 (15.7)	2 (16.7)	0.914 (0.186–4.478)	0.911
CD4 counts <200[2] (%)	57 (37.3)	3 (25.0)	0.385 (0.101–1.470)	0.162
TPHA test positive[2] (%)	108 (70.6)	10 (83.3)	2.435 (0.501–11.827)	0.270
HBV exposure[2] (%)	92 (60.1)	7 (58.3)	1.248 (0.364–4.277)	0.725
HCV Antibody positive[2] (%)	3 (2.0)	2 (16.7)	7.664 (1.369–42.890)	0.020
Extraluminal disease[2] (%)	66 (43.1)	4 (33.3)	0.559 (0.163–1.921)	0.356
No luminal agent (%)	76 (49.7)	6 (50.0)	1.070 (0.322–3.559)	0.912
Syphilis during follow-up period (%)	33 (21.6)	7 (58.3)	3.332 (0.961–11.547)	0.059

[1]Five patients died within 6 months from disease onset and their data were excluded from analysis.
[2]Status at diagnosis of IA.
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that cyst form ameba often disappears spontaneously without any treatment [28,29]. There is controversy about the need for cyst eradication following metronidazole or tinidazole treatment, especially in endemic areas where re-infection is frequent. In this study, recurrence of IA within the first year of metronidazole treatment was noted in only two patients of 82 patients who did not receive luminal therapy. Moreover, long-term follow-up indicated IA recurrence also in those who received luminal agents, and the benefits obtained from luminal treatment seemed to have disappeared. IA recurred more frequently in those with HCV infection, which was recently reported to be transmittable sexually among MSM [30], and in those who acquired new syphilis infection during the follow-up period, suggesting that sexually active MSM tend to experience IA recurrence due to re-acquisition of new *E. histolytica* infection. HBV exposure and positive TPHA at IA diagnosis did not correlate with IA recurrence probably because the high prevalence of these two parameters in this study masked the difference between recurrence and non-recurrence cases. Educational approach for safer sex may be more appropriate rather than luminal treatment to prevent IA recurrence after treatment.

Eleven genetic strains of *E. histolytica* were identified in this study and none of them had been reported so far from geographic areas other than Japan [21,22,31,32], indicating that diverse Japan-specific isolates of *E. histolytica* are already prevalent among MSM in Japan. In fact, the *E. histolytica* seropositivity rate in HIV-1-infected MSM in our clinic was as high as 17.9% in 2009 (unpublished data), which is comparable with the seropositivity rate in Japanese MSM reported more than 20 years ago [5]. Unfortunately, we could not compare the genotypes of *E. histolytica* between the incidences of the primary and recurrent IA within the same individuals due to the lack of appropriate stocked samples, which would have probably demonstrated acquisition of new infection.

Considered together, the results emphasize the difficulty of preventing IA recurrence without educational approach to prevent new amebic infection even after successful IA treatment in the high risk groups such as HIV-1-infected MSM. The spread of *E. histolytica* in MSM of other developed countries beyond Asia should be of great concern.

Supporting Information

Table S1 Patient demographics with and without luminal treatment.
(DOC)

Table S2 Genotyping data of 6 STR loci in 14 clinical samples.
(DOC)

Author Contributions

Conceived and designed the experiments: HG JT SO. Performed the experiments: KW AEdC TN. Analyzed the data: KW HG. Contributed reagents/materials/analysis tools: KW HG JT SO. Wrote the paper: KW HG.

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Impact of Small Body Weight on Tenofovir-Associated Renal Dysfunction in HIV-Infected Patients: A Retrospective Cohort Study of Japanese Patients

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Abstract

Background: Treatment with tenofovir is sometimes associated with renal dysfunction. Limited information is available on this side effect in patients with small body weight, although the use of tenofovir will spread rapidly in Asia and Africa, where patients are likely to be of smaller body weight.

Methods: In a single-center cohort, Japanese patients with HIV infection who started tenofovir-containing antiretroviral therapy were retrospectively analyzed. The incidence of tenofovir-associated renal dysfunction, defined as more than 25% decrement of estimated glomerular filtration rate (eGFR) from the baseline, was determined. The effects of small body weight and body mass index (BMI) on tenofovir-associated renal dysfunction, respectively, were estimated in univariate and multivariate Cox hazards models as the primary exposure. Other possible risk factors were evaluated by univariate analysis and those found significant were entered into the multivariate analysis.

Results: The median weight of 495 patients was 63 kg. Tenofovir-related renal dysfunction occurred in 97 (19.6%) patients (incidence: 10.5 per 100 person-years). Univariate analysis showed that the incidence of tenofovir-related renal dysfunction was significantly associated with smaller body weight and BMI, respectively (per 5 kg decrement, HR = 1.23; 95% CI, 1.10–1.37; $p<0.001$)(per 1 kg/m^2 decrement, HR = 1.14; 95% CI, 1.05–1.23; $p=0.001$). Old age, high baseline eGFR, low serum creatinine, low CD4 count, high HIV viral load, concurrent nephrotoxic drugs, hepatitis C infection, and current smoking were also associated with tenofovir-related renal dysfunction. Multivariate analysis identified small body weight as a significant risk (adjusted HR = 1.13; 95% CI, 1.01–1.27; $p=0.039$), while small BMI had marginal significance (adjusted HR = 1.07; 95% CI 1.00–1.16; $p=0.058$).

Conclusion: The incidence of tenofovir-associated renal dysfunction in Japanese patients was high. Small body weight was identified as an independent risk factor for tenofovir-associated renal dysfunction. Close monitoring of renal function is advocated for patients with small body weight treated with tenofovir.

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Introduction

Tenofovir disoproxil fumarate (TDF) is one of the most widely used nucleotide reverse transcriptase inhibitors (NRTI) for patients with HIV infection, with proven efficacy and safety [1–6]. However, TDF is known to cause renal proximal tubular dysfunction, and several case reports have been published with TDF-related Fanconi syndrome, diabetes insipidus, and acute tubular necrosis, which sometimes lead to acute renal failure [7–10]. Long-term TDF use also reduces glomerular filtration rate more than other NRTIs [11–14]. To date, the nephrotoxic effect of TDF is regarded as mild and tolerable. A recently published meta-analysis has reported that the use of TDF is associated with a statistically significant but only modest renal dysfunction, and recommended that TDF use should not be restricted even when regular monitoring of renal function and serum phosphate levels is impractical [15]. However, the TDF-related renal dysfunction 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 [16–19].

The 2010 WHO guideline on antiretroviral therapy for HIV infection in adults and adolescents, usually applied to resource-constrained settings, recommends TDF as one of the components of first line therapies (URL:http://whqlibdoc.who.int/publications/

2010/9789241599764_eng.pdf). It is expected that the use of TDF will spread rapidly in Asia and Africa in the near future, where patients are more likely to be of small body weight. Thus, at this stage, it is important to establish the relationship between TDF-associated renal dysfunction and body weight. A small body weight is considered a risk factor for TDF-associated renal dysfunction, in addition to old age, high baseline serum creatinine level, low CD4 count, concurrent use of ritonavir-boosted protease inhibitor, and concurrent use of nephrotoxic drugs [4,17,19–21]. To our knowledge, there is almost no report that primarily analyzed the influence of body weight on TDF-associated renal dysfunction. Since Japanese are generally of smaller stature and have a lower median body weight than Whites and African Americans, who mostly comprise the cohorts of studies published to date, it is important to investigate the impact of TDF-associated renal dysfunction in Japanese patients.

Based on the above background, the present study was designed to determine the incidence of TDF-associated renal dysfunction in Japanese patients and analyze the impact of small body weight on TDF-associated renal dysfunction.

Methods

Ethics Statement

This study was approved by the Human Research Ethics Committee of National Center for Global Health and Medicine (Text S1). All patients included in this study provided a written informed consent for their clinical and laboratory data to be used and be published for research purposes. This study has been conducted according to the principles expressed in the Declaration of Helsinki.

Study Design and Settings

We performed a single-center, retrospective cohort study of HIV-infected Japanese patients using 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.

Study Subjects

The study population were patients >17 years of age who commenced treatment with standard 300 mg/day of TDF-containing antiretroviral regimen at our clinic between January 1, 2002 to March 31, 2009. Both treatment-naive and patients with experience in antiretroviral treatment but not TDF, with an estimated glomerular filtration rate (eGFR) of >60 ml/min/1.73 m^2 were enrolled. Patients were followed up until September 31, 2009. Patients were excluded if their follow-up period at our facility was less than 24 weeks after commencement of TDF-based therapy, if they had started TDF at other facilities, or if there was evidence of prior TDF use. We only included Japanese patients in order to examine a population with comparatively homogenous basic demographics and background.

Measurements

Outcome measure: TDF-associated renal dysfunction. We defined TDF-associated renal dysfunction as more than 25% decrease in eGFR relative to the baseline [17,22,23]. Baseline eGFR was estimated for each patient from the average of two successive serum creatinine measurements made closest to and preceding the commencement of TDF by no more than 90 days. Changes in eGFR were plotted from the baseline measurement until the value diminished to less than 75% of the baseline or at the end of the follow-up period. The eGFR values at occurrence of TDF-associated renal dysfunction, at censoring, and closest to and preceding 24, 48, and 96 weeks to the diagnosis were collected. Patients generally visited our clinic between every month to every 3 months, and measurement of eGFR was usually conducted on every visit. eGFR was calculated using the equation from the 4-variable Modification of Diet in Renal Disease (MDRD) study [24].

Primary exposure variable. Our primary exposure variables were body weight and body mass index (BMI) at the time of commencement of TDF-containing antiretroviral therapy (ART). BMI was calculated by the equation: BMI = [body weight (kg)/height $(m)^2$].

Other variables: potential risk factors. Potential risk factors for TDF-associated renal dysfunction were determined according to previous studies and collected together with the basic demographics from the medical charts [4,19,20,25]. They included sex, age, baseline laboratory data: CD4 cell count, HIV viral load, and serum creatinine, and other medical conditions (antiretroviral treatment-naïve or experienced, concurrent ritonavir-boosted protease inhibitors, concurrent nephrotoxic drugs such as ganciclovir, sulfamethoxazole/trimethoprim, ciprofloxacin, and NSAIDs, diabetes mellitus, 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, dyslipidemia defined by current treatment with lipid-lowering agents, and current smoking) [26]. We used the data on or closest to and preceding the day of starting TDF-containing ART by no more than 90 days. The data on weight change from the baseline to the end of follow-up period and the frequency of eGFR monitoring for each patient were collected.

Statistical analysis

The time to 25% decline in eGFR from the baseline was calculated from the date of treatment initiation to the date of occurrence of TDF-associated renal dysfunction. Censored cases represented those who discontinued TDF, dropped out, referred to other facilities, or at the end of follow-up period. The time from TDF initiation to 25% decrease in eGFR was analyzed by the Kaplan Meier method for the whole cohort. To estimate the impact of body weight on the incidence of TDF-associated renal dysfunction, we calculated the impact of every 5 kg decrement from the median weight using Cox proportional hazards regression analysis. The impact of every 1 kg/m^2 decrement in BMI on the incidence of TDF-associated renal dysfunction was estimated by the same method. 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 weight on TDF-associated renal dysfunction, we conducted three models using multivariate Cox proportional hazards regression analysis. Model 1 was the aforementioned univariate analysis for every 5 kg decrement. Model 2 included sex, age 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 10 years, serum creatinine >0.8 mg/dl, CD4 count <200/µl, HIV viral load per log10/ml, concurrent nephrotoxic drugs, co-infection with hepatitis C, and current smoking). Concurrent ritonavir-boosted protease inhibitors were also added in Model 3 although their p value was 0.116 in the univariate analysis. This was based on the results of several studies suggesting that concurrent use of ritonavir-boosted protease inhibitors is a risk factor for TDF-associated renal dysfunction

[19,20]. The eGFR was excluded from multivariate analysis because of its multicollinearity with sex, age, and serum creatinine, since eGFR was gained by the equation of those variables. The impact of every 1 kg/m^2 decrement in BMI on the incidence of TDF-associated renal dysfunction was estimated by the same method with Model 1 to Model 3.

Four other analyses were conducted to further examine the relationship between low body weight and TDF-associated renal dysfunction. First, the time from initiation of TDF therapy to 25% decrease in eGFR was analyzed by the Kaplan Meier method for intertertile baseline body weight categories: <59, 59–67, and >67 kg. The log-rank test was used to determine statistical significance. Second, to investigate the impact of changes in muscle mass on changes in the eGFR as calculated by MDRD, we compared weight changes with one-way ANOVA among intertertile baseline weight categories. We also conducted the sensitivity analysis by adding the variable "weight change" in multivariate analysis. Third, the median and interquartile value for the actual fall in eGFR from the baseline to 24, 48, and 96 weeks for the whole cohort and three baseline weight categories, respectively, were calculated. The eGFR value at 24, 48, and 96 weeks included those that were censored before reaching 24, 48, and 96 weeks, respectively, so that we could interpret the data for actual fall in eGFR, including not only survived cases but also censored cases. Fourth, we counted the number of patients whose eGFR decreased to <60 and <10 ml/min/1.73 m^2, and who discontinued TDF with the clinical diagnosis of renal dysfunction due to TDF. Chi-square test was used to determine whether the difference among the weight categories was statistically significant.

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

Results

Between January 1, 2002 to March 31, 2009, 599 patients started TDF-containing ART (Figure 1). Of these, 104 patients were excluded based on the abovementioned criteria. Thus 495 patients were included in the present study (Dataset S1). Table 1 shows the demographics, laboratory data, and medical conditions of the study population at baseline. Two patients received ART with 3 NRTIs, 3 patients received ART with one protease inhibitor (PI), one non-NRTI (NNRTI), and tenofovir/emtricitabine, and the remaining patients had a standard ART with 2 NRTIs and either PI, NNRTI, or integrase inhibitor (INI). The median body weight and BMI were 63 kg and 21.9 kg/m^2, respectively. The median age of the patients was 38 years and 95.2% were males. The eGFR was well maintained (median: 120.9 ml/min/1.73 m^2), and the median baseline CD4 count was 247/μl. Of the total, 208 patients (42%) were antiretroviral treatment naïve, while 287 were treatment-experienced patients. Viral load was suppressed to <50 copies/ml in 162 (32.7%) patients. 403 (81.4%) were on concurrent PIs as the key drug, 367 (74.1%) were on ritonavir-boosted PIs, and only 83 (16.8%) had NNRTIs as the key drug. Smoking was prevalent among the study population, as 240 (48.5%) were identified as a current smoker.

TDF-associated renal dysfunction defined by more than 25% decrease of eGFR from baseline occurred in 97 patients (19.6%), with an estimated incidence of 10.5 per 100 person-years. The median time from commencement of TDF to occurrence of TDF-associated renal dysfunction was 39 weeks (IQR 13.5–99.4 weeks) (range: 1–1,841 days). The total observation period was 924.7 patient-years (median 72 weeks, IQR 38.6–139.3 weeks). Figure 2 shows the Kaplan-Meier survival curve for the occurrence of TDF-associated renal dysfunction for the whole cohort.

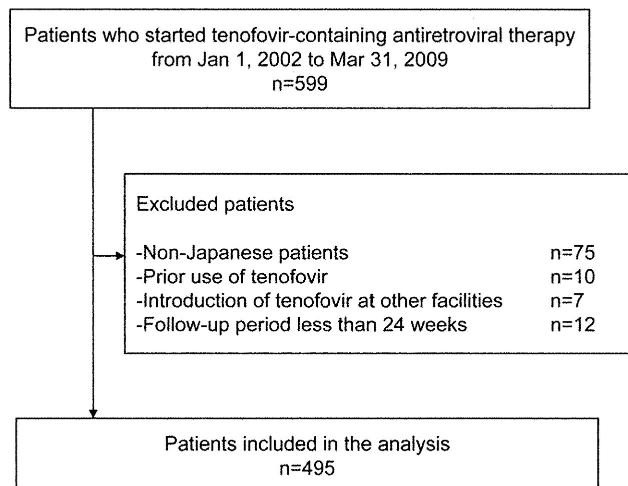

Figure 1. Flow diagram of patient selection.
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Univariate analysis showed a significant relationship between TDF-associated renal dysfunction and every 5 kg less than the median body weight (HR = 1.23; 95% CI, 1.10–1.37; p<0.001), and 1 kg/m^2 less BMI than the median BMI (HR = 1.14; 95% CI, 1.05–1.23; p = 0.001) (Table 2). Furthermore, old age, high eGFR, low serum creatinine, low CD4 counts, high HIV viral load, concurrent use of nephrotoxic drugs, presence of chronic hepatitis C, and smoking were associated with TDF-related renal dysfunction. On the other hand, concurrent use of PIs, ritonavir boosted PIs, and LPV/r tended to be associated with TDF-related renal dysfunction, albeit statistically insignificant. Treatment-naïve or Treatment-experienced was not associated with TDF-related renal dysfunction.

Multivariate analysis showed that every 5 kg less than the median body weight was a significant risk for TDF-associated renal dysfunction after adjustment for sex and age (adjusted HR = 1.21; 95% CI, 1.07–1.36; p = 0.002) (Table 3, Model 2), and also after adjustment for other risk factors (adjusted HR = 1.13; 95% CI, 1.01–1.27; p = 0.039) (Table 3, Model 3). Similarly, every 1 kg/m^2 less than the median BMI was also a significant risk factor for TDF-associated renal dysfunction even after adjustment for sex and age (adjusted HR = 1.13; 95% CI 1.05–1.22; p = 0.002) (Table 4, Model 2), and tended to be a significant factor after adjustment for other variables (adjusted HR = 1.07; 95% CI 1.00–1.16; p = 0.058) (Table 4, Model 3). Old age and current smoking were also independent risk factors in both multivariate analysis for body weight and BMI (Table 3, Model 3 and Table 4, Model 3).

In complementary analyses, First, Figure 3 shows the relation between probability of TDF-associated nephrotoxicity and time from initiation of TDF therapy to 25% decrease in eGFR analyzed by the Kaplan Meier method for intertertile baseline weight categories. Compared to patients with baseline body weight >67 kg, patients with baseline weight <59 kg were significantly more likely to develop >25% decline in eGFR (p = 0.002). On the other hand, the difference in this probability between patients with baseline weight 59–67 kg and those >67 kg was only marginally significant (p = 0.073, log-rank test). Secondly, one-way ANOVA

Table 1. Baseline demographics and laboratory data.

Characteristics		
Median (IQR) weight (kg)	63	(57–69)
Median (IQR) BMI (kg/m^2)	21.9	(20.3–23.8)
Male, n (%)	471	(95.2)
Median (IQR) age	38	(33–46)
Median (IQR) eGFR (ml/min/1.73 m^2)	120.9	(104.8–138.2)
Median (IQR) serum creatinine (mg/dl)	0.72	(0.64–0.81)
Median (IQR) CD4 count (/μl)	247	(159–371)
Median (IQR) HIV viral load (log10/ml)	3.73	(1.60–4.81)
HIV viral load <50 copies/ml, n (%)	162	(32.7)
Antiretroviral therapy naïve, n (%)	208	(42.0)
Key drugs, n (%)*		
PIs	403	(81.4)
Ritonavir-boosted PIs	367	(74.1)
LPV/r	175	(35.4)
ATV/r	131	(26.5)
FPV/r	52	(10.5)
DRV/r	9	(1.8)
FPV	14	(2.8)
ATV	4	(0.8)
NFV	15	(3)
SQV	2	(0.4)
IDV	1	(0.2)
NNRTIs	83	(16.8)
EFV	65	(13.1)
NVP	17	(3.4)
ETR	1	(0.2)
INI		
RAL	10	(2.0)
Concurrent use of nephrotoxic drug, n (%)	131	(26.5)
Diabetes mellitus, n (%)	30	(6.1)
Hepatitis B, n (%)	75	(15.2)
Hepatitis C, n (%)	52	(10.5)
Hypertension, n (%)	28	(5.7)
Dyslipidemia, n (%)	40	(8.1)
Smoking, n (%)	240	(48.5)
Median (IQR) weight change (kg)	0.0	(−2.0–2.25)
Median (IQR) frequency of eGFR monitoring	16	(9.0–27)

(n = 495).
*Two patients did not take any key drugs. Three patients took both PI and NNRTI.
IQR: interquartile range, BMI: body mass index, eGFR: estimated glomerular filtration rate, PI: protease inhibitor, LPV/r: lopinavir/ritonavir, ATV: atazanavir, FPV: fosamprenavir, DRV: darunavir, NFV: nelfinavir, SQV: saquinavir, IDV: indinavir, NNRTI: non-nucleos(t)ide reverse transcriptase inhibitor, EFV: efavirenz, NVP: nevirapine, ETR: etravirine, INI: integrase inhibitor, RAL: raltegravir.
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showed that weight changes among the three baseline weight categories were not significantly different (p = 0.206). Sensitivity analysis after adding the variable "weight change" in Model 3 multivariate analysis (Table 3) showed that adjusted hazard ratio for weight per 5 kg decrement hardly changed (adjusted HR 1.131; 95% CI, 1.007–1.271; p = 0.038). Thirdly, Table 5 shows the median and interquartile values for the actual falls in eGFR from the baseline to 24, 48, and 96 weeks. The eGFR decreased gradually in all categories, except for patients with baseline weight

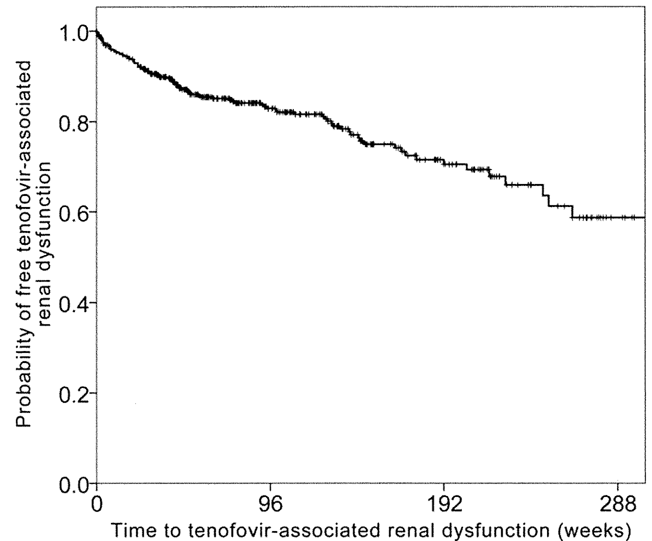

Figure 2. Kaplan-Meier curve showing the time to 25% reduction in eGFR for the whole cohort. eGFR: estimated glomerular filtration rate.
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Table 2. Univariate analysis for TDF-associated renal dysfunction.

	HR	95%CI	P value
Weight per 5 kg decrement	1.23	1.10–1.37	<0.001
BMI per 1 kg/m^2 decrement	1.14	1.05–1.23	0.001
Male gender	0.54	0.26–1.11	0.094
Age per 10 years	1.22	1.02–1.45	0.027
eGFR per 10 ml/min/1.73 m^2	1.10	1.05–1.15	<0.001
Serum creatinine >0.8 mg/dl	0.51	0.30–0.88	0.014
CD4 count <200/μl	1.97	1.32–2.93	0.001
HIV viral load per log10/ml	1.15	1.01–1.30	0.037
Antiretroviral therapy naïve	0.98	0.63–1.52	0.927
Concurrent key drugs			
Any PIs	1.52	0.89–2.59	0.124
Ritonavir boosted PIs	1.46	0.91–2.33	0.116
LPV/r	1.45	0.97–2.17	0.072
ATV/r	1.05	0.66–1.68	0.826
Concurrent nephrotoxic drug	1.59	1.04–2.42	0.031
Diabetes mellitus	1.57	0.76–3.24	0.220
Hepatitis B	1.36	0.82–2.24	0.231
Hepatitis C	1.80	1.07–3.04	0.028
Hypertension	1.18	0.51–2.69	0.702
Dyslipidemia	0.97	0.47–2.00	0.932
Smoking	1.57	1.05–2.36	0.028

TDF: tenofovir, HR: hazard ratio, CI: confidence interval, BMI: body mass index, eGFR: estimated glomerular filtration rate, PI: protease inhibitor, LPV/r: lopinavir/ritonavir, ATV: atazanavir.
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