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Analysis of the Hepatic Functional Reserve, Portal Hypertension, and Prognosis of Patients With Human Immunodeficiency Virus/Hepatitis C Virus Coinfection Through Contaminated Blood Products in Japan

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ABSTRACT

Background. As the survival of human immunodeficiency virus (HIV)-infected individuals has improved due to the widespread use of antiretroviral therapy, the mortality rate due to hepatitis C virus (HCV)-related liver disease has increased in HIV/HCV-coinfected patients.

Aim. The aims of this study were to establish the appropriate therapeutic strategy for HIV/HCV-coinfected patients by evaluating the liver function, including the hepatic functional reserve and portal hypertension, and to investigate the prognosis of HIV/HCV-coinfected patients in Japan.

Patients and Methods. In addition to regular liver function tests, the hepatic functional reserve of 41 patients with HIV/HCV coinfection was evaluated using the indocyanine green retention rate and liver galactosyl serum albumin-scintigraphy. The data for 146 patients with HIV/HCV coinfection through blood products were extracted from 4 major HIV centers in Japan. In addition to liver function tests, the platelet counts (PLT) were evaluated as a marker of portal hypertension.

Results. In spite of the relatively preserved general liver function test results, approximately 40% of the HIV/HCV-coinfected patients had an impaired hepatic functional reserve. In addition, while the albumin and bilirubin levels were normal, the PLT was <150,000/μL in 17 patients. Compared with HCV mono-infected patients with a PLT <150,000/μL, the survival of HIV/HCV-coinfected patients was shorter (HCV, 5 years, 97%; 10 years, 86% and HIV/HCV, 5 years, 87%; 10 years, 73%; $P < .05$).

Conclusion. These results must be taken into account to establish an optimal therapeutic strategy, including the appropriate timing of liver transplantation in HIV/HCV-coinfected patients in Japan.

FROM 1970 until the early 1980s, blood products were imported to Japan, and contaminated blood products were unknowingly used to treat patients with hemophilia. It was later revealed that these patients were sometimes infected with both human immunodeficiency virus (HIV) and hepatitis C virus (HCV; HIV/HCV coinfection) [1].

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However, as the survival of HIV-infected people has improved due to the widespread use of antiretroviral therapy, the mortality due to HCV-related liver disease has increased in HIV/HCV-coinfected patients [2,3].

The main aims of this investigation were to investigate the status of portal hypertension and the prognosis in HIV/HCV-coinfected patients, and to establish an appropriate therapeutic strategy for HIV/HCV-coinfected patients, including the timing of liver transplantation, in Japan.

PATIENTS AND METHODS

Routine hematology and blood chemistry tests (general liver function), abdominal ultrasonography, and contrast-enhanced computed tomography (CT) were performed for 30 patients with HIV/HCV coinfection at Nagasaki University Hospital. To investigate the hepatic functional reserve, liver GSA-scintigraphy and the indocyamine green retention test at 15 minutes were performed. In addition, upper gastrointestinal tract endoscopy to diagnose gastroesophageal varices was performed.

The data of the 146 patients who had acquired HIV/HCV coinfection through blood products were extracted from 4 major HIV centers in Japan, including the AIDS Clinical Center, Osaka National Hospital, Yokohama Municipal Hospital, and Kyushu Medical Center. In addition to liver function tests, platelet counts (PLT) were evaluated as a marker of portal hypertension. As a control, HCV mono-infected patients from Nagasaki Medical Center were used for comparison.

RESULTS

In spite of the relatively well-maintained general liver functions, approximately 40% of the HIV/HCV-coinfected patients had an impaired hepatic functional reserve (Table 1). In addition, in spite of maintained albumin and bilirubin levels, the PLT was <150,000/μL in 17 coinfected patients, indicating the presence of ongoing portal hypertension.

Even with Child-Pugh A liver function, the HIV/HCV-coinfected patients showed a worse prognosis than the HCV mono-infected patients. The prognosis was especially poor in those with lower PLT than in the patients with a normal PLT (Table 2). When compared with HCV mono-infected patients with a PLT <150,000 μL, the survival of HIV/HCV-coinfected patients was much shorter (HCV, 5 years, 97%; 10 years, 86% and HIV/HCV, 5 years, 87%; 10 years, 73%; $P < .05$).

Table 1. Patient Characteristics

Child-Pugh A/B/C	38 (93%)/1 (2%)/2 (5%)
ICG R15 (%)	
<10/10–20/20–30/30<	24 (59%)/8 (20%)/3 (7%)/6 (14%)
GSA schincigram LHL15	
>0.9/0.8–0.9/0.8>	28 (69%)/6 (15%)/7 (16%)
Liver configuration on CT	
Normal/CH/LC	10 (24%)/17 (42%)/14 (34%)
Splenomegaly	
Yes/no	26 (63%)/15 (37%)
Esophageal varices	
Yes/no	13 (32%)/28 (68%)

CH, chromic hepatitics; LC, liver cirrhosis.

Table 2. Patient Survival after Diagnosis

	5Y OS	10Y OS	
HCV mono-infection (Child-Pugh A)	97%	86%	
HIV/HCV coinfection (Child-Pugh A)			
PLT > 150,000	94%	85%	
PLT < 150,000	87%	73%	$P < .05$ vs HCV mono-infection

5Y OS, 5 year patient survival; 10Y OS, 10 year patient survival.

DISCUSSION

In HIV/HCV-coinfected patients, liver failure due to HCV hepatitis was previously reported to be enhanced by antiretroviral therapy ART-related hepatotoxicity, especially manifesting as noncirrhotic portal hypertension (NCPH) [4,5]. One of the ART drugs, Didanosin (DDI), has been suspected to be related to the serious morbidity observed in coinfected patients [6]. Thus, not only in patients with deteriorated liver function, such as in Child-Pugh B or C cases, but also even in Class A cases, the patients' liver function can easily deteriorate abruptly [7]. The natural course of pure NCPH is unknown because it can be modulated by HCV or other causes, and has only been reported as case series. An important study of "NCPH in HIV Mono-Infected Patients Without HCV" was published in 2012 [8]. All 5 patients had portal hypertensive symptoms, such as ascites or variceal bleeding, after receiving antiretroviral therapy.

Therefore, all HIV/HCV-coinfected patients should be carefully followed up so as not to miss an opportunity for liver transplantation (LT) [9]. The prognosis for HIV/HCV-coinfected patients was reported to be worse than that for HCV mono-infected patients [10]. In the present study, coinfected patients with a PTL <150,000 μL had an especially poor prognosis, with a shorter survival than mono-infected patients. Our results should be taken into account to establish a therapeutic strategy, while also considering the appropriate timing of LT in HIV/HCV-coinfected patients.

In 2013, based on the evidence of rapid progression of the liver cirrhosis and portal hypertension in patients with HIV/HCV coinfection, a rank-up system for the waiting list for deceased donor LT was set up in Japan. Even HIV/HCV-coinfected liver cirrhotic patients with Child-Pugh class A can be listed for LT as "point 3" because of the NCPH (noncirrhotic portal hypertension) nature. Coinfected patients with Child-Pugh class B and C disease can be listed as "point 6" and "point 8," respectively, based on the data collected by the HIV/acquired immunodeficiency syndrome (AIDS) project team of the Ministry of Health, Labor, and Welfare of Japan, and the published literature [11]. This primarily covers victims who received contaminated blood products for hemophilia.

Future perspectives on LT for HIV/HCV coinfection include the following: new anti-HCV agents should be

developed to improve the control against HCV; new ART drugs, such as Raltegravir, should facilitate post-transplantation immunosuppressive therapy; noninvasive tests for portal hypertension, such as the fibroscan, should be performed for hemophilic patients; and the development of guidelines for the management hemophilia in the peri-operative period should facilitate better outcomes.

In conclusion, the present results should be taken into account to establish an optimal therapeutic strategy, including the appropriate timing of LT in HIV/HCV-coinfected patients.

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Short communication

Raltegravir and elvitegravir-resistance mutation E92Q affects HLA-B*40:02-restricted HIV-1-specific CTL recognition

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Abstract

Interplay between drug-resistance mutations in CTL epitopes and HIV-1-specific CTLs may influence the control of HIV-1 viremia. However, the effect of integrase inhibitor (INI)-resistance mutations on the CTL recognition has not been reported. We here investigated the effect of a raltegravir and elvitegravir-resistance mutation (E92Q) on HLA-B*40:02-restricted Int92-102 (EL11: ETGQETAYFLL)-specific CTLs. EL11-specific CTLs recognized E92Q peptide-pulsed and E92Q mutant virus-infected cells less effectively than EL11 peptide-pulsed and wild-type virus-infected cells, respectively. *Ex vivo* ELISpot analysis showed no induction of E92Q-specific T cells in chronically HIV-1-infected individuals. Thus, we demonstrated that EL11-specific CTL recognition was affected by the INI-resistance mutation.

Keywords: Integrase inhibitor; CTL; HIV

1. Introduction

Treatment with effective antiretroviral therapy (ART) results in a decline in viral load and increase in $CD4^+$ T cell count in the majority of HIV-1-infected individuals [1]; whereas the presence of drug-resistance mutations can contribute to increased risk of virologic failure [2]. Many such mutations occur in regions of HIV-1 Pol, which encompasses a number of previously identified cytotoxic T lymphocyte (CTL) epitopes [3,4]. If drug-resistance mutations enhance the immunogenic antigenicity of the CTL epitope, drug treatment might drive the CTL response towards HIV control; otherwise, they may cause an immunologically uncontrollable HIV infection if they affect the CTL responses. On the other hand, HIV-1-specific CTLs, especially those against HIV-1 Gag and Pol, play a major role in controlling replication of HIV-1 [5,6]. However, HIV-1 escapes from the host immune system by various mechanisms [7]. The appearance of CTL escape mutations is one of them [5,6]. If such CTL escape mutations occur in the drug-target proteins, including reverse transcriptase, protease, and integrase, they may alter the drug sensitivity or modify the patterns of drug-resistance mutations [8,9].

Several studies have demonstrated CTL responses to HIV-1 drug-resistance mutations. Some protease inhibitor (PI)-resistance mutations (G48V, M46I, I47A, and I50V) abolish CTL recognition [10]; whereas other PI-resistance mutations (L63P and L10I) enhance it [4]. HIV-1 viruses with the nucleoside/nucleotide reverse transcriptase inhibitor (NRTI)-resistance M184V mutation show reduced viral replication capacity compared to the wild-type virus; whereas individuals having an M184V-specific CTL response have a lower viral load than those without this CTL response [11], suggesting that M184V-specific CTLs may suppress the replication of this mutant

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HIV-1. Thus, the M184V mutation may have the effect of controlling HIV-1. Most NRTI-resistance mutations (M41L, L74V, M184V, and T215Y/F) do not impair CTL recognition [3]. In contrast, T cells fail to recognize wild type but effectively recognize the non-nucleoside reverse-transcriptase inhibitors (NNRTIs)-resistance mutation K103N in some patients [12], indicating that some drug-resistance mutations have a positive effect on CTL recognition. Thus, drug-resistance mutations have various effects on CTL recognition. The combined effect of CTL escape mutations I135T/L/R and drug-resistance mutation E138K confers significant resistance towards NNRTIs, though separately they have only a mild resistance effect toward NNRTI [9], suggesting that CTL pressure sometimes generates more potent drug-resistance mutations.

Raltegravir (RAL), the first integrase inhibitor (INI), was approved in 2007, followed by elvitegravir (EVG) in 2012; although EVG shows extensive cross-resistance with RAL. Both RAL and EVG are important options for first-line therapy as well as for the treatment of highly ART-experienced patients. INIs can suppress virus replication in HIV-1 patients harboring viruses resistance to other ARTs and constitute a valuable option for salvage therapy. INIs have a relatively low genetic barrier to resistance; and a single mutation is able to confer resistance to INIs [13]. Despite the potency, tolerability, and durability of INIs, signature resistance mutations against RAL (N155H, Q148H/K/R, Y143C/H/R, E92Q, and a few others) were detected in 60% of patients who experienced virologic failure in clinical trials involving highly treatment-experienced patients [14]. Moreover, the most common EVG-resistance mutations that emerged in clinical trials were E92Q, Q148R/H/K, and N155H [15]. E92Q alone reduces susceptibility to EVG more than 20 fold and causes limited (<5 fold) cross resistance to RAL [16]. As these drugs are frequently used to treat HIV-1 patients, and resistance mutations are appearing in clinical isolates, analysis of the interaction between CTL and INI-resistance mutations is important in studies concerning the effect of drug-resistance mutations on immune-recognition. In the present study, we investigated the effect of the INI-resistance mutation E92Q on EL11-specific HLA-B*40:02-restricted CTL recognition in chronically HIV-1-infected Japanese individuals having HLA-B*40:02 (The frequency of this allele is 16.6% in Japan).

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. Plasma and peripheral blood mononuclear cells (PBMCs) were separated from heparinized whole blood. The HLA type of the patients was determined by standard sequence-based genotyping. Five HLA-B*40:02^{+} chronically HIV-1-infected individuals were recruited for this study.

2.2. Synthetic peptides

INI-resistance peptide EL11–E92Q was 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

C1R cells expressing HLA-B*40:02 (C1R–B*4002), 721.221 cells expressing CD4 (721.221-CD4), and 721.221-CD4 cells expressing HLA-B*40:02 (721.221-CD4-B*4002) were previously generated [17]. These cells were maintained in RPMI 1640 medium supplemented with 10% FCS and 2.0 mg/ml hygromycin B.

2.4. 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 ml 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*40:02 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 intracellular cytokine staining 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.

2.5. HIV-1 clones

An HIV-1 mutant (NL-432-E92Q) was generated by introducing the EL11–E92Q mutation into NL-432, which is an infectious proviral clone of HIV-1. Site-directed mutagenesis (Invitrogen) based on overlap extension was used for the generation of this virus.

2.6. CTL assay for target cells pulsed with HIV-1 peptide

Cytotoxic activity was measured by the standard ^{51}Cr-release assay, as previously described [6]. Target cells (2×10^5) were incubated for 1 h with 100 μl of Ci $Na_2^{51}CrO_4$ in saline and then washed 3 times with RPMI 1640 medium containing 10% FCS. Labeled target cells (2×10^3/well) were added to 96-well round-bottomed microtiter plates (Nunc) along with the appropriate amount of the corresponding peptide. After 1 h of incubation, effector cells were added; and then incubation was carried out for 4 h at 37 °C. The supernatants were collected and analyzed with a gamma counter. Spontaneous ^{51}Cr release was determined by measuring the

counts per minute (cpm) in supernatants from wells containing only target cells (cpm spn). Maximum ^{51}Cr 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 (cpm exp − cpm spn)/(cpm max − cpm spn) × 100, where "cpm exp" is the counts per minute in the supernatant in the wells containing both target and effector cells. Relative specific lysis of peptide-pulsed target cells was defined as (specific lysis of exp − specific lysis of cells without peptide).

2.7. CTL assay for target cells infected with HIV-1

721.221-CD4-B*40:02 cells were exposed to NL-432 or NL-432-E92Q viruses, and 721.221-CD4 cells were exposed to NL-432, for several days. The cells were used as target cells for CTL assays once approximately 40–60% of the cells had been infected, which were confirmed by intracellular staining for HIV-1 p24 antigen. Infected cells were labeled with ^{51}Cr as described above. Labeled target cells were added along with effector cells into round-bottomed microtiter plates (Nunc), and the mixtures were incubated for 6 h at 37 °C. Relative specific lysis of target cells infected with HIV-1was defined as (specific lysis of exp − specific lysis of uninfected cells)/(percentage of infected cell used).

2.8. ELISpot assay

The appropriate amount of EL11 or EL11–E92Q peptides and PBMCs from HLA-B*40:02$^+$ individuals chronically infected with HIV-1 were added to 96-well polyvinylidene plates (Millipore, Bedford, MA) that had been pre-coated with 5 mg/mL anti-IFN-γ mAb 1-D1K (Mabtech, Stockholm, Sweden). The plates were incubated for 16 h at 37 °C in 5% CO_2 and then washed with PBS before the addition of biotinylated anti-IFN-γ mAb (Mabtech) at 1 mg/mL. After the plates had been incubated at room temperature for 90 min, they were subsequently incubated with streptavidin-conjugated alkaline phosphatase (Mabtech) for 60 min at room temperature. Individual cytokine-producing cells were detected as dark spots after a 20-min reaction with 5-bromo-4-chloro-3-idolyl phosphate and nitro blue tetrazolium by using an alkaline phosphatase-conjugate substrate (Bio-Rad, Richmond, CA, USA). The spot number was counted by using an Eliphoto-Counter (Minerva Teck, Tokyo, Japan). The CD8$^+$ T cells without peptide stimulation were used as a negative control.

2.9. Sequencing of plasma RNA

Viral RNA was extracted from the plasma of HIV-1-infected individuals by using a QIAamp MinElute Virus Spin Kit (QIAGEN). cDNA was synthesized from the RNA with SuperScript III First-Strand Synthesis System for RT-PCR and random primer (Invitrogen). HIV-1 Pol gene was amplified by nested PCR using Taq polymerase (Promega). Sequencing reactions were performed with a Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and analyzed by use of an ABI 3500 genetic analyzer.

3. Results

3.1. INI-resistance E92Q mutation affects recognition of EL11-specific CTLs

The INI-resistance E92Q mutation is located in the HLA-B*40:02-restricted EL11 (ETGQETAYFLL) CTL epitope [17]. We therefore examined whether this mutation would affect the recognition by EL11-specific CTLs. The EL11-specific CTL clone and line, which were generated from an HIV-1-infected HLA-B*40:02$^+$ individual, effectively killed the wild-type peptide-pulsed C1R-B*40:02 cells, whereas they showed a reduced ability to kill the target cells pulsed with the E92Q mutant peptide (Fig. 1). To clarify the recognition of E92Q mutant-infected cells by EL11-specific CTLs, we generated an HIV-1 mutant virus by introducing the E92Q mutation into NL-432 (NL-432-E92Q) and then examined whether EL11-specific CTLs could kill target cells infected with the E92Q mutant virus. The EL11-specific CTL clone and cell line killed both WT-infected target cells and the NL-432-E92Q-infected ones, though the killing activity of the clone and cell line for the latter cells was significantly reduced as compared with that for the former ones (Fig. 2). These results indicate that INI-resistance E92Q mutation reduced EL11-specific CTL recognition.

3.2. Ex vivo CD8$^+$ T-cells fail to recognize EL11–E92Q peptide

To clarify the *ex vivo* CD8$^+$ T cell response to EL11-E92Q, we measured the responses in 5 HLA-B*40:02$^+$ individuals chronically infected with HIV-1 by performing ELISpot assays using EL11 and EL11-E92Q peptides. A strong T cell

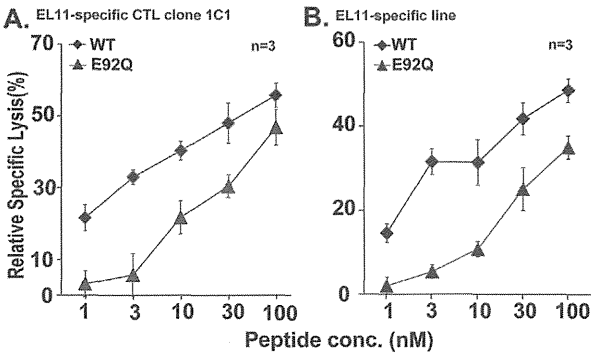

Fig. 1. Relative cytolytic activity of EL11-specific CTLs against target cells pulsed with the WT (EL11) or mutant (EL11–E92Q) peptide. An EL11-specific CTL clone and cell line were generated from PBMCs by stimulating them with WT peptide from a HLA-B*40:02 patient, KI-400, infected with WT virus. The antiviral activities of an EL11-specific CTL clone 1C1 (A) and the EL11-specific cell line (B) were analyzed. Relative cytotoxic activity toward C1R-B*40:02 cells prepulsed with the WT or EL11-E92Q peptide at concentrations of 1–100 nM was measured. The cytotoxic activity assay was performed at an E:T ratio of 1:1. The error bars indicate standard deviations.

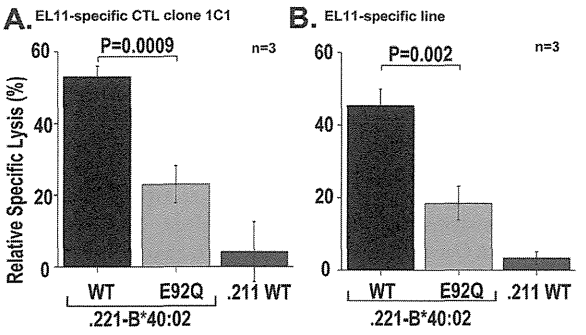

Fig. 2. Relative cytolytic activity of EL11-specific CTLs against target cells infected with WT (NL-432) or mutant virus (NL432-E92Q). Relative cytotoxic activity against 721.221-CD4-B*40:02 cells infected with NL-432 (WT virus) or NL-432-E92Q (E92Q virus) was assessed. (A) WT virus-infected (43.7% of total cells were p24 Ag^+) and E92Q virus-infected (59.1% of total cells were p24 Ag^+) cells were used as target cells. (B) WT virus-infected (59.2% of total cells were p24 Ag^+) and E92Q virus-infected (44.5% of total cells were p24 Ag^+) cells were used as target cells. NL-432 virus-infected 721.221-$CD4^+$ (.221 WT) cells were used as a negative control. The antiviral activities of an EL11-specific CTL clone, 1C1 (A), and an EL11-specific cell line (B) were analyzed. The cytotoxic activity was measured at E:T ratio of 1:1. The error bars indicate standard deviations. *P* values were determined by using Student's *T*-test.

response to the EL11 peptide was found in 4 of the 5 individuals (Fig. 3). However, these individuals did not have any response to the EL11–E92Q peptide. These results indicate that the HLA-B*40:02$^+$ individuals failed to recognize the INI-resistance E92Q mutation *ex vivo*.

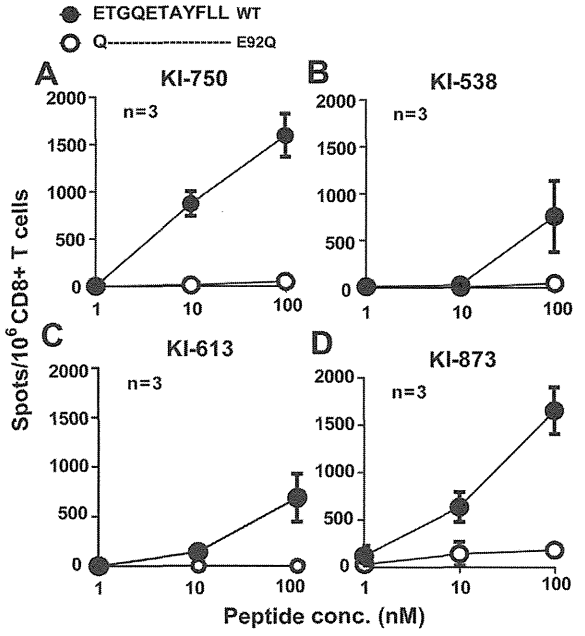

Fig. 3. *Ex vivo* $CD8^+$ T-cells response specific for WT (EL11) or mutant (EL11–E92Q) peptides in chronically HIV-1-infected HLA-B*40:02$^+$ individuals. $CD8^+$ T cell responses to EL11 or EL11–E92Q peptides by PBMCs from 5 HLA-B*40:02$^+$ chronically HIV-1-infected individuals were analyzed by performing ELISpot assays using WT (EL11) or mutant (E92Q) peptides at 1 nM–100 nM. A response eliciting greater than 200 spots was taken as a positive response. Four of the 5 individuals showed positive responses to the E11 peptide but not to the mutant one.

3.3. Frequency of the INI-resistance E92Q mutation in treatment-naïve Japanese individuals

The E92Q mutation was reported to occur in 2.2% of INI-treated patients, whereas the mutation was not found in treatment-naïve ones [18]. To clarify the accumulation of the E92Q mutation in Japanese individuals, we analyzed this part of the HIV-1 sequence in 363 treatment-naïve chronically HIV-1-infected Japanese patients and found no E92Q mutation in these individuals (data not shown). Thus, so far the E92Q mutation has not accumulated in the Japanese population.

4. Discussion

The RAL and EVG-resistance E92Q mutation is accumulating in INI-treated HIV-1 clade B patients [18]. In contrast, INI-resistance mutations, which are currently clinically relevant, are absent or highly infrequent in INI treatment-naïve patients [19]. We presently analyzed the HIV-1 sequence in 363 Japanese treatment-naïve patients and did not observe this INI-resistance E92Q mutation in any members of this population. These observations indicate that this mutation has not yet accumulated in treatment-naïve patients. INI-resistance E92Q was reported to be associated with significant fitness cost [20]. It could easily revert to the wild type in the absence of drug pressure after transmission from INI-containing ART-failing patients to untreated ones. However, INIs are relatively new; and the frequency of INI-resistance mutations might increase with future extensive use of such drugs.

Mutations in the anchor residues of a peptide can diminish the binding affinity for HLA class I molecules by changing the conformation of the peptide, though non-anchor residues also have an important role in peptide binding to HLA molecules [21]. HLA-B*40:02 binding peptide has an anchor residue at P2 [22]. INI-resistance E92Q mutations occur in the non-anchor P1 position of the EL11 epitope. The INI pressure replacing glutamic acid (E), having a negatively charged side chain, in the P1 position with glutamine (Q), having a polar uncharged side chain, might change the epitope conformation, resulting in weakened peptide binding to HLA-B*40:02 or impaired antigen presentation [23]. Changes in the non-anchor P1 position of a peptide might lead to significant unfavorable contacts with residues of the TCR.

We observed that EL11-specific CTLs killed both wild-type virus-infected cells and E92Q mutant virus-infected ones *in vitro*, although the CTLs killed more effectively the former cells than the latter cells. In contrast, EL11-specific $CD8^+$ T cells failed to recognize the mutation *ex vivo*. As *ex vivo* data reflects more closely the *in vivo* than the *in vitro* data, these results suggest that EL11–E92Q epitope would not be recognized by T cells *in vivo*. CTLs have high antigen sensitivity towards EL11 [17]; and $CD8^+$ T cells specific for the EL11 epitope were detected in 4 of the 5 HLA-B*40:02$^+$ individuals examined, indicating EL11 to be an immunodominant epitope. However, since the INI-resistance EL11-E92Q epitope may not be recognized by the $CD8^+$ T cells *in vivo*,

this INI-resistance mutation could hamper the eradication of HIV-1.

In the present study, we observed the effect of an INI-resistance mutation on CTL recognition. The data presented here demonstrated that the INI-resistance E92Q mutation affected EL11-specific $CD8^+$ T cell recognition both *in vitro* and *ex vivo* in the HLA-B*40:02$^+$ individuals. The CTL epitope having incorporated this drug-resistance mutation showed reduced immunogenicity, suggesting that this INI-resistance mutation affected HIV-1 control by the CTLs. Virologic failure in RAL-containing ART is associated with integrase mutations in at least 3 genetic pathways (Q148H/K/R, N155H, and Y143R/H/C pathways), N155H pathway includes the E92Q mutation [24]. Considering that RAL-resistance pathways at failure are not predicted by baseline viral mutations, host immune pressure could be one of the determinants of the resistance pathway [25]. In order to answer the question as to whether the INI-resistance E92Q mutation can emerge more frequently in HLA-B*40:02$^+$ individuals than in those negative for it during INI-containing ART, large clinical trials may be necessary. Further research is warranted.

Conflict of interest

The authors have no conflicting financial interests.

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Note

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Thermal Stability of Tenofovir Disoproxil Fumarate in Suspension

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A previous study examined the stability of anti-HIV agents prepared according to a simple suspension method. The results indicated a significant decline in the percentage of tenofovir disoproxil fumarate (TDF) remaining when suspended and kept in a warm bath at 80 °C for 60 min. The study identified a probable decomposition product and examined the effects of the temperature and duration of suspension. The present study determined changes in the amount of TDF remaining over time when the drug was suspended at temperatures of 70 – 90 °C and kept in a warm water bath. In the thermal decomposition of TDF, the reaction rate increased as the storage temperature rose, and the remaining product was decreased following the first-order reaction. And the activation energy (Ea) was calculated as 78.6 kJ・mol^{-1} from an Arrhenius plot. The percentage of drug remaining reached 98% in 58.7 min at 55 °C. And the decomposition product, which was identified as tenofovir monoester, is the hydrolysis product of TDF. Such detailed data on the thermal stability of the TDF was considered to be useful basic data when examining the feasibility of a simple suspension method.

Key words —— simple suspension method, anti-retroviral therapy, HIV, thermal stability, tenofovir disoproxil fumarate

Introduction

The establishment of anti-retroviral therapy (ART) to treat human immunodeficiency virus (HIV) infection has improved the long-term prognosis.[1, 2] However, ART may necessitate long-term care or death may result from some other severe illnesses indicative of acquired immune deficiency syndrome (AIDS), including progressive multifocal leukoencephalopathy.

Most anti-HIV agents are taken orally. When patients have difficulty swallowing the drugs, they must be administered via a gastric tube, nasogastric tube, or similar device. This has led to an examination of the effects of drug grinding or suspension. Grinding may lead to drug loss during preparation and risks exposing the pharmacist to teratogenic drugs. In addition, unstable drugs cannot be prescribed in long-term doses after grinding, which leads to increased patient visits. A simple suspension method avoids the drawbacks of grinding, but issues with stability of the principal drug may arise when it is suspended in warm water. Because drugs must continue to be administered accurately and reliably for ART to be successful, poorly managed administration can

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quickly cause HIV to become resistant.[3,4] A previous study examined the stability of active drugs by high-performance liquid chromatography (HPLC) when commonly used anti-HIV agents were suspended using a simple suspension method.[5] Single agents or two or more agents were disintegrated and suspended in warm water at 55, 60, 70, or 80 °C, and the percentage of active principle drug remaining was determined after 20 min at room temperature by HPLC. No significant decline in the percentage of the drugs remaining was noted. In contrast, the percentage of tenofovir disoproxil fumarate (TDF) remaining decline significantly when it was suspended and kept in a warm bath at 80 °C for 60 min and a new peak with the almost same percentage, presumed to be a decomposition product, was detected.[5] To assess the thermal stability of TDF in detail, in this study we examined the effects of the suspension temperature and storage time on changes in the active principle remaining over time in accelerated conditions. In addition, we attempted to identify the peak presumed decomposition product of TDF when the drug was suspended in high temperature.

Materials and Methods

1. Drug

TDF (300 mg Viread® tablets) was purchased from Torii Pharmaceutical Co, Ltd (Tokyo, Japan).

2. Reagents

Ethyl *p*-hydroxybenzoate, used as the internal standard, and methanol were purchased from Nacalai Tesque, Inc, Kyoto, Japan. Sodium dihydrogen phosphate was purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan).

3. TDF reference solution

Three tablets were used to prepare the reference solution. Each tablet was crushed several times with a pestle and then added to a 250 mL polypropylene volumetric flask to which 20 mL water were added. After completely disintegrating the tablets, 10 mL of the internal standard (12.8 mg/mL) solution and methanol were added to a volume of 250 mL. Twenty milliliters of this solution were again diluted in methanol to a final volume of 100 mL. The solution was filtered with a 0.8 μm cellulose acetate membrane filter (Toyo Roshi Kaisha, Ltd, Tokyo, Japan) and 10 μL were used for HPLC-ultra-violet (UV) analysis.

4. Effect of suspension temperature on TDF degradation

(1) Changes in the amount of drug remaining at various temperatures

The sample solutions, as described above, were suspended at different temperatures and kept in a warm bath. The percentage of drug remaining was measured by HPLC-UV after a given period of suspension. An Arrhenius plot was constructed using the degradation rate constant, and activation energy (Ea) was calculated based on the Arrhenius equation ①. When the drug was suspended in water at 55 ℃, the times required for the percentage of drug remaining to reach 98% and 50% were calculated based on the equation.

$$\log k = \log A - Ea/2.303RT \quad ①$$

where k is the first-order degradation rate constant, A is the frequency factor, R is the gas constant, and T is the absolute temperature.

(2) HPLC conditions

HPLC was performed using a Waters 717 Autosampler, Waters 1525 Binary HPLC Pump, and Waters 486 Tunable Absorbance Detector with an Inertsil ODS-3 4.6 × 250 mm column

(GL Science Inc, Tokyo, Japan) at 25 ℃. Recording and analysis of measured data were done using Empower™2 (Nihon Waters KK, Tokyo, Japan) analytical software. The injection volume was 10 μL, the duration of analysis was 15 min, the flow rate was 1.5 mL/min from 0–3.5 min and 1.8 mL/min from 3.5–15 min, and the mobile phase was a mixture (85:15) of methanol and 0.025 M sodium dihydrogen phosphate. Ethyl *p*-hydroxybenzoate served as the internal standard and was detected at 265 nm.[6)]

(3) Calculation of the percentage of drug remaining

The peak height ratio of the sample prepared using the simple suspension method for the peak height of the internal standard (A) to that the peak height ratio of the sample for the reference standard (B) was determined. The residual percentage of TDF was calculated using the following equation:

$$\text{Residual TDF}\,(\%) = A/B \times 100$$

5. Identification of the TDF decomposition product

(1) Identification of the degradation peak in high-temperature suspensions

Suspensions were prepared as described above, kept in a water bath at 90 °C for 60 min, and then analyzed by liquid chromatography- high resolution mass spectrometry (LC/HRMS).

The measured accurate mass of molecular ion for degradation product was measured using HRMS and compared with the calculated exact mass of the possible molecular formulas.

(2) LC/HRMS conditions

①Chromatography

Chromatographic separations were performed on a $5C_{18}$-MS-II 1.6 × 50 mm column maintained at 25 °C (Nacalai Tesque, Inc) using an Ultimate 3000 HPLC system (Thermo Fisher Scientific Inc, Massachusetts, USA). The mobile phase used was a mixture (60:40) of 0.01 M ammonium formate and methanol. The sample injection volume was 10 μL and the total analysis duration was 40 min at a flow rate of 0.4 mL/min. The column eluent was directed to the mass spectrometer.

②Mass spectrometry

HRMS was performed on an LTQ Orbitrap Discovery mass spectrometer (Thermo Fisher Scientific Inc) operating in positive ion mode with an electrospray source. The sheath gas was set to 50 (arbitrary units) and the auxiliary gas was set to 10 (arbitrary units) at a capillary temperature of 330 °C. The capillary voltage and spray voltage were set to 25 V and 5.0 kV, respectively. The instrument was operated in full-scan mode from m/z 100–1000 at a 50,000 resolving power. The mass spectrometer was mass calibrated just prior to starting the injection sequence.

Results

1. Effect of temperature on drug decomposition

Changes in the amount of drug remaining at different temperatures over time are shown in **Fig 1** and **Table 1**. A decline in the percentage of drug remaining over time was evident at all temperatures and in a temperature-dependent manner, with 93.3% remaining after 60 min at 70 °C, 90.1% remaining at 75 °C, 82.2% remaining at 80 °C, and 74.1% remaining at 90 °C. Straight

Table 1 Degradation rate constant (min^{-1}) at various suspension temperatures

Suspension Temperature (℃)	Degradation rate constant (min^{-1})
70	1.272×10^{-3}
75	1.447×10^{-3}
80	3.213×10^{-3}
90	5.219×10^{-3}

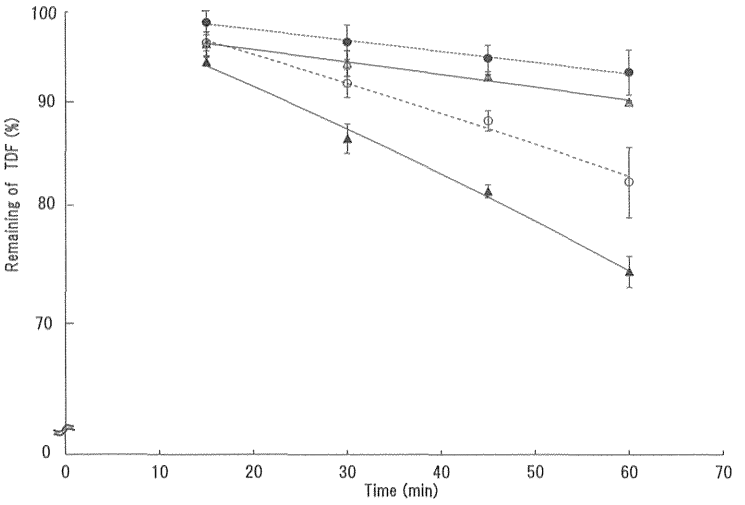

Fig 1 Changes in the average percentage of TDF remaining with various temperatures of suspension

Temperature of suspension: ● 70 °C, Δ 75 °C, ○ 80 °C, ▲ 90 °C. Different lines are approximate straight lines when using the first-order reaction rate equation with various suspension temperatures, (n = 3).

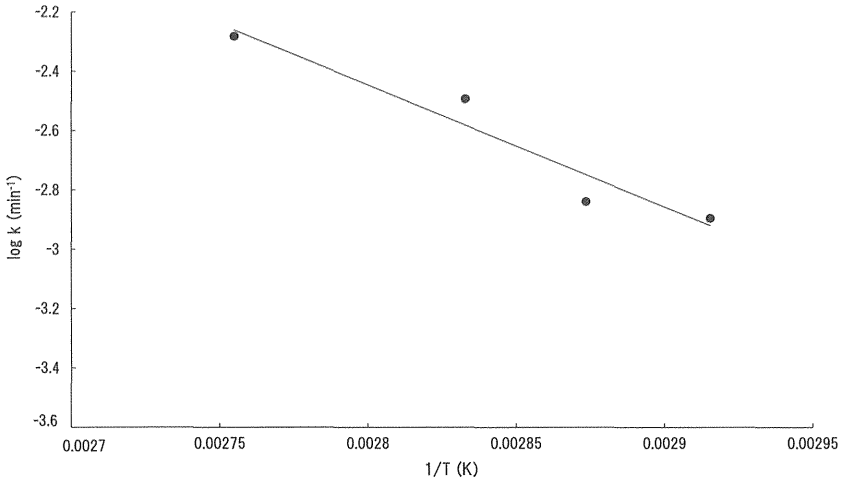

Fig 2 Arrhenius plot

Regression equation log k = − 4104(1/T) + 9.046, r = 0.9656. k: first-order reaction rate constant, T: absolute temperature (K).

lines obtained for each temperature, suggesting that decomposition of TDF proceeded in accordance with the first-order reaction rate equation.

An Arrhenius plot of the reaction rate constant, determined based on the regression equation in **Fig 1**, yielded an approximate straight line (**Fig 2**). The Ea for TDF degradation was approximately 78.6 kJ · mol^{-1}. When the simple suspension method was used, the first-order reaction rate constant (k) at 55 °C was calculated to be 3.41 × 10^{-4} (min^{-1}). The time for the percentage of drug remaining to reach 98% was 58.7 min and to reach 50% was 33 h and 50 min.

2. Identification of degradation products after storage

The results of LC-HRMS analysis of the reference and sample solution after 60 min storage at 90 °C are shown in **Figs 3 and 4**. A peak unlike that for the reference was detected at 3.32 min.

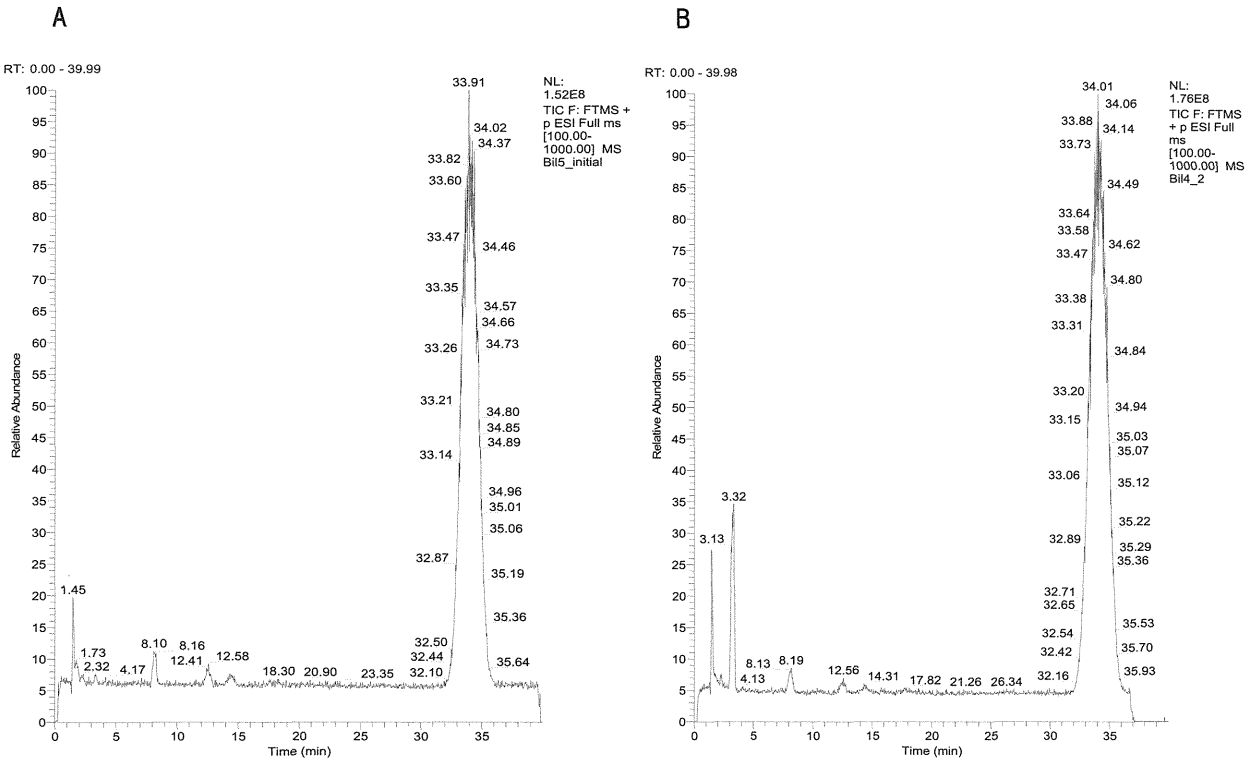

Fig 3 MS chromatograms for the reference sample and after a 60 min suspension at 90 °C
A:The reference standard , B:Stored at 90 °C for 60 min.

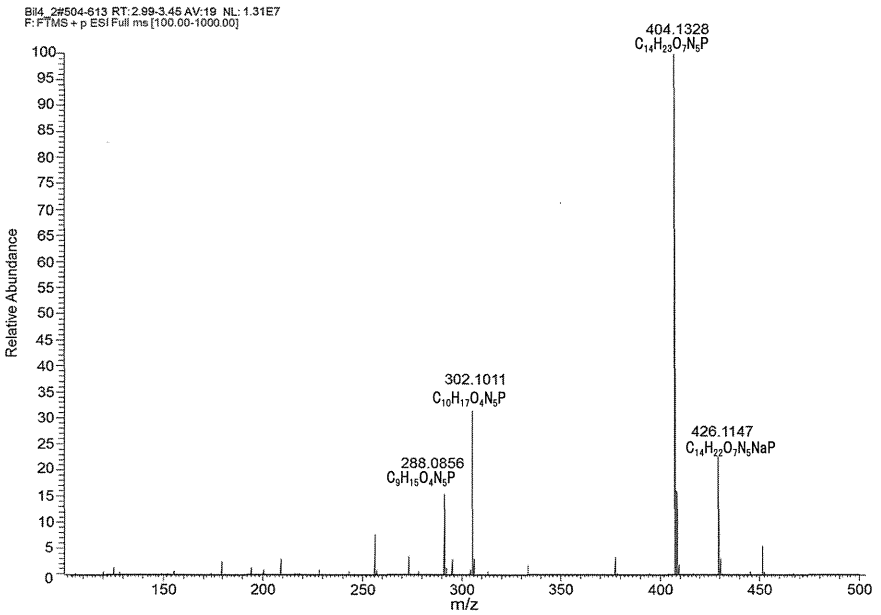

Fig 4 Mass spectrum of a decomposition product after 60 min storage at 90 °C

The other degradation products were not detected in HPLC-UV and LC/HRMS chromatograms. The difference between the theoretical value of 404.1330 $[M+1]^+$ (*m/z*), $C_{14}H_{23}O_7N_5P$ and the measured value of 404.1328 $[M+1]^+$ (*m/z*) for degradation products was −0.2 mmu. This led to identification of the decomposition product as a tenofovir (TFV) monoester (**Fig 5**), which was also shown in results from interview form long-term storage test and acceleration test.[7)]

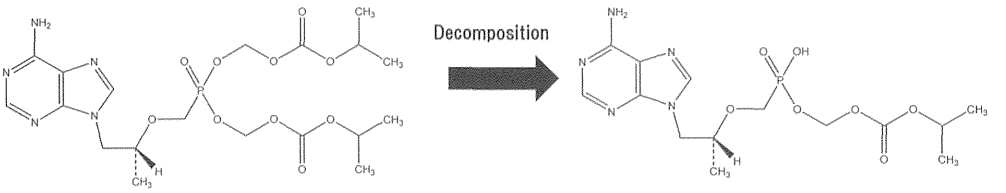

Fig 5 The scheme of the thermal decomposition of TDF after 60 min storage at 90 °C
A: TDF, B: TFV monoester.

Discussion

During the thermal decomposition of TDF, the reaction rate increased as the storage temperature rose, and the remaining decreased following the first-order reaction. In a first-order reaction, the remaining decreases exponentially with respect to the reaction time, and the temperature dependence of the reaction rate becomes prominent when Ea is shown to be large. For this reason, a rapid drop in TDF content is concerned when suspended inadvertently at a high temperature, and therefore, the Ea computation was conducted using an Arrhenius plot. The result obtained was 78.6 kJ · mol^{-1}, which was within the range of Ea values observed in decomposition of pharmaceutical substances[8-10] (40–130 kJ · mol^{-1}). In addition, a decomposition product was identified as the monoester form based on its precise mass and molecular formula (**Fig 5**), which was also shown in results from interview form long-term storage test and acceleration test, suggesting that the hydrolysis reaction has occurred. Given that the activation energy was comparable to the values reported for hydrolysis of other drugs, the temperature dependence of the degradation rate may be considered to be comparable with the other drugs.

TDF is an oral prodrug diesterified to improve the bioavailability (BA) of TFV in oral administration. The BA of the monoester, which was detected in this study as a product from TDF decomposition, has not been reported, although the BA of TDF and TFV in animal experiments has been reported to be 37.8% and 17.8%, respectively. This indicates that the BA of TFV was approximately half of TDF.[11] Because TDF is promptly conversion into TFV through a monoester,[12] the monoester is considered an intermediate that does not affect safety. The presence of only monoester peaks in HPLC-UV and LC/HRMS chromatograms indicated that the hydrolysis reaction was not progressing to TFV. When dividing powder medicine, the permissible error is ± 10% of the average weight. Thus, the decomposition of TDF deemed to be clinically satisfactory as it was <10% of the starting material. In addition, the therapeutic effect is unlikely to be affected when TDF is suspended at the temperature used in clinical practice because a 2% decrease in the remaining compound takes approximately 60 min at 55 °C. Furthermore, the remaining compound is >95%, even when suspended at 80 °C and left at the same temperature for 15 min, although prompt use after suspension is still desirable.

TDF is a nucleotide analogue reverse transcriptase inhibitor that has exceptional antiviral action and is well tolerated. It is the drug of choice in international principle guidelines for HIV-1 infection and is a key treatment recommended by the US Department of Health and Human Services (The Department of Health and Human Services.

Guidelines for the Use of Antiretroviral Agents in HIV-1-Infected Adults and Adolescents, 2013 : revised on February 12, http://aidsinfo.nih.gov/, 2013.7.31). The effectiveness of TDF against HIV is related to the area under the blood concentration-time curve.[13] Thus, if the percentage of drug remaining declines as a result of suspension, resistance may develop. In addition, TDF also acts against the hepatitis B virus[14] such that a lack of effectiveness in cases of HIV/HBV co-infection could render the patient HIV- and HBV-drug resistant.

Assuming the simple suspension method is used in actual clinical practice, suspension may be done incorrectly at temperatures of 55 °C or higher. Such detailed data on the thermal stability of the TDF was considered to be a useful basic data when examining the feasibility of a simple suspension method.

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