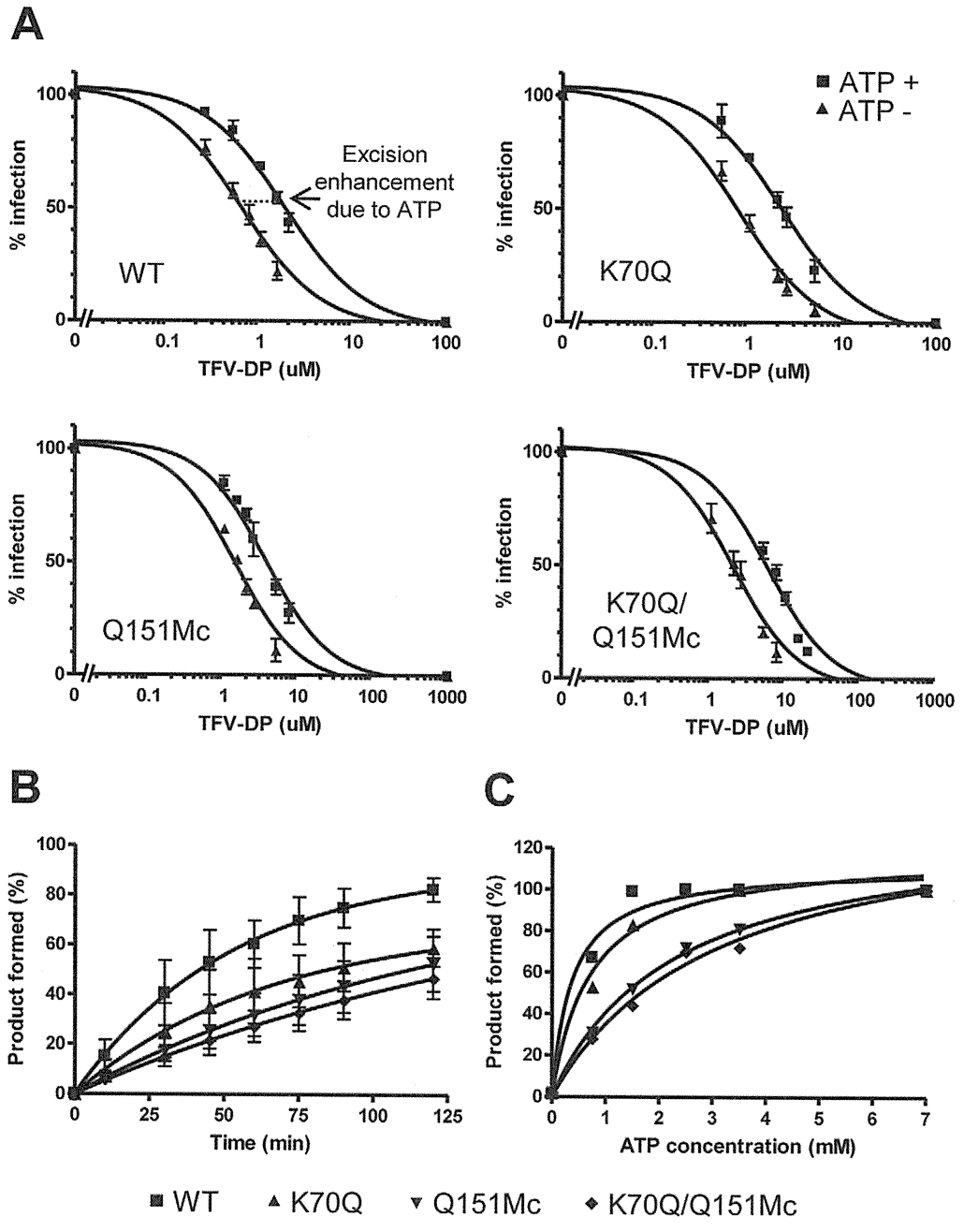

Figure 3. Effects of RT mutations K70Q, Q151Mc, or K70Q/Q151Mc on DNA primer extension activity and on ATP-based excision activities. (A) Effect of varying concentrations of TFV-DP on the primer extension activities of HIV-1 WT and mutant RTs. The experiments were carried out in the presence (■) or absence (▲) of 3.5 mM ATP (B) Time dependence of ATP-based rescue of TFV-terminated primers. TFV-terminated T_{31}/P_{18} oligos (20 nM) were incubated with 60 nM RT and 3.5 mM ATP. The reaction mixture also included excess of competing dATP (100 μM) that prevented reincorporation of TFV-DP and 0.5 μM dTTP, and 10 μM ddGTP that allowed extension of the rescued primer by two nucleotides and chain termination. Rescue products (WT [■], K70Q [▲], Q151Mc [▼] and K70Q/Q151Mc [♦]) were analyzed at indicated time points. (C) ATP-based rescue was dependent on concentration of ATP. Reactions were as in (B), but for 30 minutes and at varying concentrations of ATP. Rescue products at 7 mM ATP are defined as 100% product formed.
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Q151Mc, and K70Q/Q151Mc RTs (the apparent $K_{D\text{-}ATP}$ for WT, K70Q, Q151Mc and K70Q/Q151Mc were 0.4, 0.7, 2.3, and 3.1 mM, respectively), suggesting that a better binding of ATP may contribute to the slightly enhanced excision activity of WT RT (Fig. 3C and Figure S2C). Collectively, these results rule out the possibility that K70Q/Q151Mc becomes resistant to TFV through the excision mechanism.

Pre-Steady Kinetic Constants for Binding and Incorporation of dATP and TFV-DP

To determine whether the resistance by K70Q/Q151Mc is caused by an increased preference of physiological dATP substrate over TFV-DP, we carried out pre-steady state transient kinetic analyses of WT, K70Q, Q151Mc, and K70Q/Q151Mc enzymes. The kinetic constants $k_{pol\text{-}dATP}$ and $K_{D\text{-}dATP}$ for WT and mutant

enzymes are presented in Table 2 and Fig. 4 (and also in Figure S3). The results reveal that K70Q, Q151Mc, and K70Q/Q151Mc RTs have increased $k_{pol\text{-}dATP}$ as well as $K_{D\text{-}dATP}$. Both Q151Mc and K70Q/Q151Mc enzymes incorporate dATP faster than WT (17.9 and 14.6 s^{-1}, respectively *vs.* 6.3 s^{-1}) but have a weaker binding affinity for dATP than WT RT (5.4 and 5.0 μM, respectively *vs.* 2.6 μM). Hence, the catalytic efficiency ratio of dATP incorporation remains similar for all enzymes ($k_{pol\text{-}dATP}/K_{D\text{-}dATP}$ ratios for WT, K70Q, Q151Mc, and K70Q/Q151Mc were 2.4, 2.2, 3.3, and 2.9 $\mu M^{-1}\cdot s^{-1}$, respectively). On the contrary, a significant change in the incorporation efficiency of TFV was observed. The K70Q and K70Q/Q151Mc enzymes had more than 4.5-fold reduced affinity for TFV than the WT enzyme ($K_{D\text{-}TFV}$ values were 8.6 and 8.9 μM compared to 1.9 μM). In addition, the turnover rates of TFV incorporation by the WT and K70Q enzymes were comparable ($k_{pol\text{-}TFV}$ were 2.8 and 3.1 s^{-1}, respectively). The addition of the K70Q mutation to Q151Mc also reduced the k_{pol} for TFV-DP. The net effect of these changes was a significant reduction in the TFV-DP incorporation efficiencies of the mutant enzymes compared to the WT enzyme ($k_{pol\text{-}TFV}/K_{D\text{-}TFV}$ ratios for WT, K70Q, Q151Mc, and K70Q/Q151Mc were 1.47, 0.36, 0.3, and 0.11 $\mu M^{-1}\cdot s^{-1}$, respectively; Table 2). WT RT incorporated TFV-DP most efficiently, followed by K70Q>Q151Mc>K70Q/Q151Mc enzymes. As a direct measure of the enzyme's ability to discriminate between the natural dATP substrate and the TFV, we determined the "selectivity", defined as the ratio of efficiency of the enzyme to incorporate dATP over TFV-DP ($k_{pol\text{-}dATP}/K_{D\text{-}dATP}/k_{pol\text{-}TFV}/K_{D\text{-}TFV}$). The selectivity values demonstrate that the K70Q/Q151Mc enzyme favors incorporation of dNTP over TFV-DP 26.3 times compared to 1.6 times by the WT enzyme, leading to a 16.4-fold resistance to TFV (defined as $selectivity_{mutant}/selectivity_{WT}$; Table 2). This resistance is more than twice the TFV resistance of Q151Mc and 4 times the TFV resistance of K70Q.

Molecular modeling

Molecular dynamics simulations on the control structural coordinates of the WT RT/DNA/TFV-DP crystal structure [45] did not cause any significant structural changes, suggesting that the modeling protocols do not alter the structures in ways that are not related to the K70Q or Q151Mc mutations. The root mean square deviation (rmsd) between the Cα atoms of the WT structures before and after simulation was 0.1 Å. Similarly, the rmsd between the Cα atoms of WT and mutant RT molecular models were also very low (~0.1 Å). Comparison of these models showed a significant repositioning of residue 65 in Q151Mc/K70Q (Fig. 5), and to a lesser extent in K70Q or Q151Mc RTs (not shown). Additional smaller changes in the side chains of residues 151, 70, and 72 were also observed (Fig. 5). The structure of TFV-DP was also slightly adjusted, possibly as a result of the changes in the surrounding residues (Fig. 5). While residue 70 is located proximal to residue 65, and to the phosphates of the incoming TFV-DP, it does not appear to interact directly with these structural elements.

Discussion

We have discovered a novel HIV mutation that causes high-level resistance to TFV-DF. We have also determined the biochemical mechanism of this resistance. TFV-DF is a valuable NRTI therapeutic option for patients infected with multi-drug resistant Q151Mc HIV-1 [22]. We demonstrate here that Q151Mc can acquire an additional mutation, K70Q, which expands the multi-drug resistance to include high-level resistance to TFV-DF. We identified this mutant during genotypic analysis of clinical isolates from an HIV-infected patient who was not responding to TFV-DF. The K70Q/Q151Mc set of mutations is currently rare among HIV-infected patients. However, we believe that similar to K65R, its prevalence will increase, as tenofovir use continues to rise. Our virological studies with recombinant viruses confirmed that the observed enhancement and expansion of multi-drug resistance is the consequence of the addition of K70Q to Q151Mc HIV. Recently, the concept of clinical cut-offs (CCOs) has been introduced to improve the prediction of drug resistance during antiretroviral therapies. CCOs are better correlated with virologic response than biological cut-offs [51,52]. Importantly, K70Q/Q151Mc is 10 times less susceptible to TFV-DF than WT HIV-1, whereas the CCOs for TFV-DF is defined as a 2.1-fold reduction in virologic response to this inhibitor. Moreover, K70Q/Q151Mc is at least twice as resistant to TFV as the well-known TFV-resistant K65R in the background of Q151Mc (as reported in the Stanford HIV Drug Resistance Database).

Previous studies have offered insights into the drug resistance mechanism of similar mutations (K70E, K70G, K70R, and K70T). Specifically, K70E was selected in patients with virological failure after TFV-DF-based antiviral therapy [53,54,55]. K70T emerged in the background of Q151Mc during *in vitro* selection by TFV-DF [56]. K70R is a key mutation involved in resistance to AZT and appears in the background of other excision enhancement mutations [2,3,57]. In our case, a new mutation (K70Q) was

Table 2. Pre-steady state kinetic constants for binding and incorporation of dATP and TFV-DP by WT, K70Q, Q151Mc and K70Q/Q151Mc HIV-1 RT.

Pre-steady state kinetic constants[a]								
Enzyme[b]	dATP			TFV-DP			Selectivity[c]	Resistance[d]
	k_{pol} (s^{-1})	K_d (μM)	k_{pol}/K_d ($\mu M^{-1}\cdot s^{-1}$)	k_{pol} (s^{-1})	K_d (μM)	k_{pol}/K_d ($\mu M^{-1}\cdot s^{-1}$)		
WT	6.3±0.5	2.6±0.1	2.4±0.2	2.8±0.08	1.9±0.2	1.47±0.07	1.6	-
K70Q	8.4±0.4	3.8±0.6	2.2±0.4	3.1±0.4	8.6±1.5	0.36±0.08	6.1	3.8
Q151Mc	17.9±0.4	5.4±0.5	3.3±0.3	1.3±0.03	4.3±0.8	0.3±0.06	11	6.9
K70Q/Q151Mc	14.6±1.6	5.0±0.07	2.9±0.3	1.0±0.03	8.9±2.1	0.11±0.03	26.3	16.4

[a]Data are means ± standard deviations from at least three independent experiments.
[b]The sequence of HIV RT WT and mutant derived from BH10.
[c]Selectivity is defined as $(k_{pol}/K_d)_{dATP}/(k_{pol}/K_d)_{TFV\text{-}DP}$.
[d]Resistance (fold) is calculated as $selectivity_{mutant}/selectivity_{WT}$.
doi:10.1371/journal.pone.0016242.t002

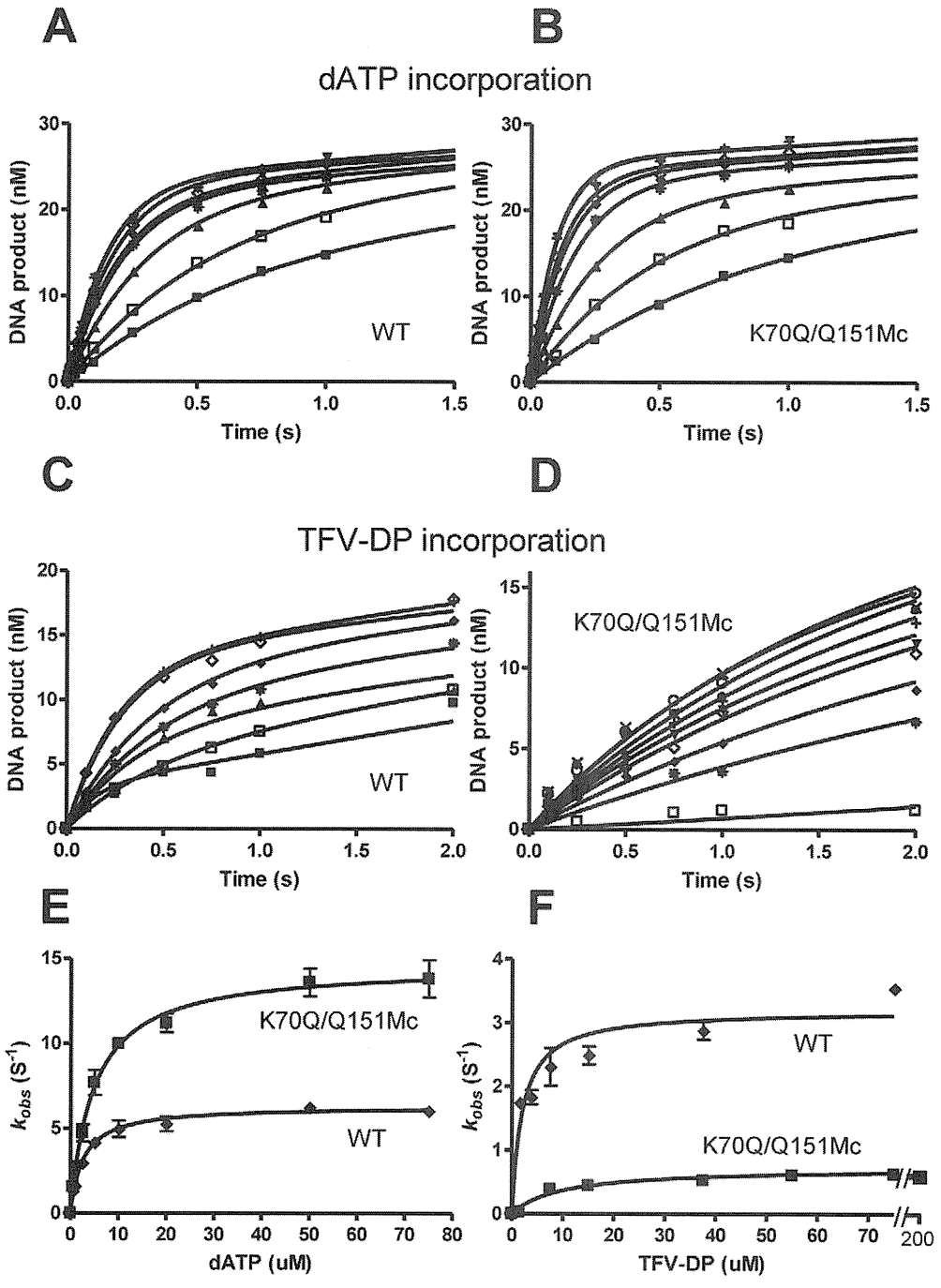

Figure 4. Pre-steady state kinetics of incorporation of dATP or TFV-DP by WT and K70Q/Q151Mc HIV-1 RTs. Single-nucleotide incorporation of dATP (panels A, B, and E) or TFV-DP (panels C, D, and F) by WT (panels A, C, E, and F) and K70Q/Q151Mc (panels B, D, E, and F). Formation of extended primer products in the reactions with WT RT and K70Q/Q151Mc RT were measured at 5 ms to 5 s time points, using the following dATP concentrations: 0.5 (■), 1 (□), 2.5 (▲), 5 (*), 10 (♦), 20 (◇), 50 (▼) and 75 μM (+). Incorporation of TFV was measured at 0.1–10 s reactions and at the following TFV-DP concentrations: 0.75 (■), 1.5 (□), 3.75 (▲), 7.5 (*), 15 (♦), 37.5 (◇) and 75 μM (+) for reactions with WT RT (panel C), and 1.5 (□), 7.5 (*), 15 (♦), 37.5 (◇), 55 (▼), 75 (+), 112.5 (•), 150 (○) and 200 μM (x) for reactions with K70Q/Q151Mc RT (panel D). (E) The amplitudes of the burst phases from the dATP reactions shown in panels A (WT, [♦]) and B (K70Q/Q151Mc, [■]) were plotted as a function of dATP concentrations. (F) The amplitudes of the burst phases from the TFV-DP reactions shown in panels C (WT, [♦]) and D (K70Q/Q151Mc, [■]) were plotted as a function of TFV-DP concentrations. The solid lines in panels A, B, C, and D represent the best fit of data to the burst equation. Each point represents the average values of three experiments.
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identified in a patient infected with Q151Mc HIV-1 during the course of TFV-DF-based antiviral therapy. The International AIDS Society-USA publishes [58] every year a list of HIV-1 drug resistance mutations compiled by a panel of experts charged with the goal of delivering accurate, unbiased, and evidence-based information for use by HIV clinical practitioners. In order for a novel mutation to be accepted in the list it should meet at least *one* of the following criteria: 1) *in vitro* passage experiments or

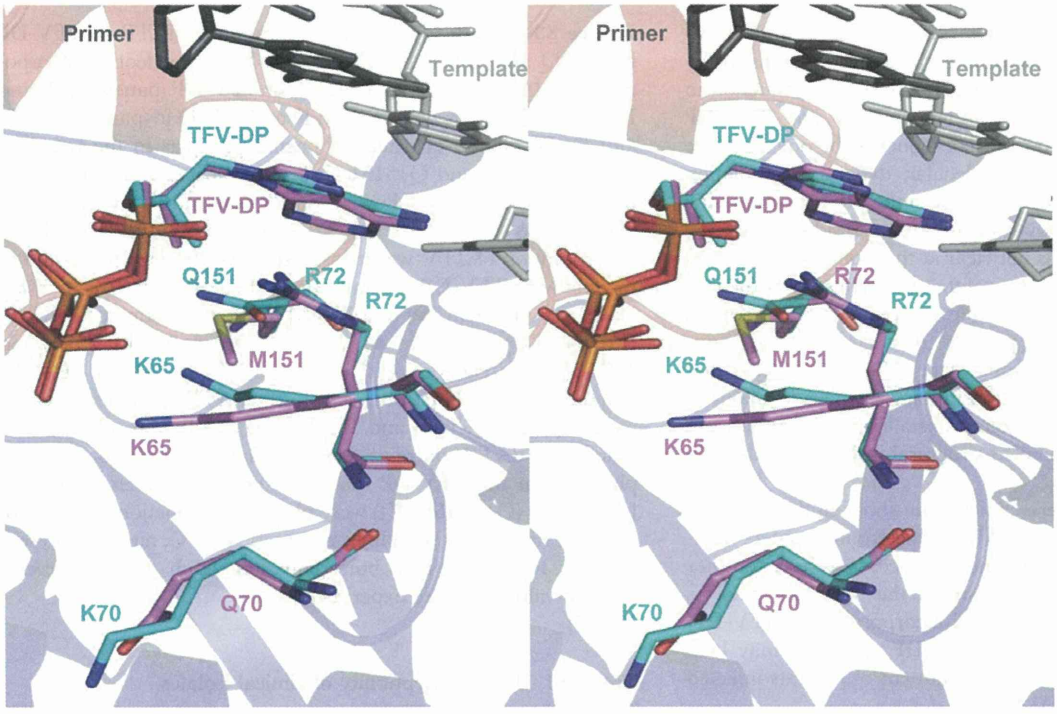

Figure 5. Stereo view of TFV-DP in the polymerase active site of WT RT and K70Q/Q151Mc RT. WT RT residues are shown as cyan sticks, K70Q/Q151Mc RT residues are shown as purple sticks. The primer strand is shown as dark gray sticks, template strand as light gray sticks. The fingers and palm subdomains are shown as blue and red cartoons, respectively.
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validation of contribution to resistance by using site-directed mutagenesis; 2) susceptibility testing of laboratory or clinical isolates; 3) nucleotide sequencing of viruses from patients in whom the drug is failing; 4) correlation studies between genotype at baseline and virologic response in patients exposed to a drug. Our study has unambiguously demonstrated that K70Q meets at least the first three criteria: evidence for criterion #1 is shown in Figure 2; for criterion #2 in Figures 1 and 2; and for criterion #3 in Figure 1 and Figure S1. Therefore, the K70Q mutation meets the criteria of a clinically relevant mutation.

In addition to the clinical and virological studies, we used biochemical techniques to determine the mechanism of TFV resistance imparted by the K70Q mutation to Q151Mc RTs. We used primer extension assays to show that K70Q/Q151Mc RT is less susceptible to TFV-DP than WT and Q151Mc RTs. We demonstrated that the mechanism of this resistance is not based on excision. On the contrary, we showed that the ATP-based excision of the mutant enzymes was slightly decreased with respect to WT RT, possibly because of decreased affinity of the mutant enzymes for the ATP excision substrate, incurred by changes in the binding environment of ATP, such as the loss of lysine at position 70.

Using transient-state kinetics we unambiguously established that the overall mechanism of K70Q/Q151Mc resistance to TFV is due to enhanced discrimination between the natural dATP substrate and TFV-DP. While all mutant enzymes had comparable efficiency of dATP incorporation, they displayed varying affinity and turnover rates of incorporation. It appears that the stronger effect of the enhanced discrimination overcomes the slight increase in sensitivity due to the small increase in excision. As a result, the mutant enzymes are resistant to the inhibitor.

Mutations at position 70 of RT have been known to confer NRTI resistance by two distinct mechanisms: K70R combined with at least two excision enhancing mutations, D67N and T215Y, enhances ATP-mediated excision of AZT and d4T [1,2,3,48] (*excision–dependent mechanism*). On the other hand, K70E causes resistance to 3TC, TFV, and ABC by lowering the maximum rate of inhibitor incorporation by RT (k_{pol}*-dependent exclusion mechanism*) [55]. Our results establish that in the background of Q151Mc, K70Q causes TFV resistance through a third mechanism: by decreasing the binding affinity of the inhibitor (K_d*-dependent exclusion mechanism*). Taken together, these findings highlight the remarkable ability of RT to use separate mutations at a single position to acquire NRTI resistance through three different mechanisms.

Our cell-based assays with infectious HIV-1 show that Q151Mc remains susceptible to TFV-DF, a finding consistent with previous reports [22]. Similarly, clinical isolates deposited at the Stanford HIV resistance database and carrying the Q151Mc mutation were also susceptible to TFV-DF, unless they also had the K65R mutation. However, pre-steady state characterization of TFV-DP incorporation by Q151Mc in this work (Table 2) and by others [59] showed that Q151Mc is less susceptible to TFV-DP than WT RT. This small discrepancy may be the result of potential differences in DNA-dependent and RNA-dependent DNA synthesis, or the result of the slightly increased excision of Q151Mc RT compared to WT RT (Fig. 3B and C).

To gain insights into the possible structural changes caused by the addition of K70Q to Q151Mc, we compared the molecular model of K70Q/Q151Mc RT/DNA/TFV-DP with the crystal structure of WT RT/DNA/TFV-DP [45] (Fig. 5). The network of hydrogen bonds involving the side-chains of K65, R72, and Q151 in the WT structure [26,27,54], is disrupted in the mutant structure. Also, Q151M and associated mutations A62V, V75I, and F77L are likely to modify the hydrophobic core of the fingers. We and others have previously shown that the side-chains of residues 72 and 65 interact with each other [35] and with Q151

and the α- and γ-phosphates of the incoming dNTP [26] or TFV-DP [45]. The functions of these residues have been established by several biochemical studies [21,25,60,61,62,63]. The reduction in polymerase rate (k_{pol}) and in binding affinity for TFV-DP (increased $K_{d.TFV\text{-}DP}$) may be the consequence of one or more such structural changes. Our molecular dynamics simulation experiments suggested a re-arrangement in the position of the side chain of K65, which is a catalytically important residue. While the precise effect of this change is not clear at this point, such changes could influence the overall binding of the substrate and/or the rate of nucleotide incorporation. Moreover, such movement of K65 in the presence of a mutation at position 70 is consistent with our previously reported crystallographic data, which established that there is an interplay between the positioning of the side chains at positions 70 and 65 [64]. Ongoing crystallographic studies are expected to provide more detailed structural insights into the role of K70Q in drug resistance.

In summary, we report here clinical data showing that addition of the K70Q mutation to the Q151Mc background confers high-level HIV resistance to TFV-DF and enhances resistance to other NRTIs. The biochemical mechanism of the TFV resistance is based on reduced binding affinity and incorporation of TFV-DP. Detection of this novel pattern of TFV-DF resistance may help adjust therapeutic regimens for the treatment of patients infected with multi-drug resistant HIV-1.

Supporting Information

Figure S1 Amino acid sequence alignment of the RT regions (amino acid 32 to 560) of the clinical isolates at time points 1 to 2 (see Figure 1A).
(DOC)

Figure S2 Effects of RT mutations K70Q, Q151Mc, or K70Q/Q151Mc on DNA primer extension activity and on ATP-based excision activities. (A) Effect of varying concentrations of TFV-DP on the primer extension activities of HIV-1 WT and mutant RTs. The experiments were carried out in the presence and absence of 3.5 mM ATP (marked as ATP (+) and ATP (−), respectively). Addition of ATP in the polymerization mixture allows measurement of the net sum of DNA polymerization and ATP-based excision activities. (B) Time dependence of ATP-based rescue of TFV-terminated primers. (C) ATP-based rescue was dependent on concentration of ATP.
(PPTX)

Figure S3 Pre-steady state incorporation of dATP or TFV-DP by K70Q and Q151Mc HIV-1 RTs. Single-nucleotide incorporation of dATP (panels A, B, and E) or TFV-DP (panels C, D, and F) by K70Q (panels A, C, E, and F) and Q151Mc (panels B, D, E, and F). Formation of extended primer products in the reactions with K70Q RT and Q151Mc RT were measured at 5 ms to 5 s time points, using the following dATP concentrations: 0.5 (■), 1 (□), 2.5 (▲), 5 (*), 10 (♦), 20 (◇), 50 (▼) and 75 μM (+). Incorporation of TFV was measured at 0.1–10 s reactions and at the following TFV-DP concentrations: 0.75 (■), 1.5 (□), 3.75 (▲), 7.5 (*), 15 (♦), 37.5 (◇) and 75 μM (▼) for reactions with K70Q RT (panel C), and 3.75 (▲), 7.5 (*), 37.5 (◇), 55 (▼), 75 (+) and 112.5 (•) for reactions with Q151Mc RT (panel D). (E) The amplitudes of the burst phases from the dATP reactions shown in panels A (K70Q, [▲]) and B (Q151Mc, [▼]) were plotted as a function of dATP concentrations. (F) The amplitudes of the burst phases from the TFV-DP reactions shown in panels C (K70Q, [▲]) and D (Q151Mc, [▼]) were plotted as a function of TFV-DP concentrations. The solid lines in panels A, B, C, and D represent the best fit of data to a burst equation. Each point represents average values of three experiments.
(PPTX)

Table S1 Drug susceptibility of clinical isolates.
(DOC)

Table S2 Drug susceptibility of HIV-1 variants carrying mutation at residue 70.
(DOC)

Table S3 Drug susceptibility of HIV-1 variants carrying mutation at residue 70 in the background of Q151M complex.
(DOC)

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Author Contributions

Conceived and designed the experiments: AH ENK SO SGS. Performed the experiments: AH MMS KAK EM YS KS. Analyzed the data: AH MMS KAK KS SGS. Contributed reagents/materials/analysis tools: AH ENK SGS OS. Wrote the paper: AH ENK KS SGS.

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BRIEF ARTICLE

Diagnostic value of antigenemia assay for cytomegalovirus gastrointestinal disease in immunocompromised patients

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Abstract

AIM: To investigate the utility of the cytomegalovirus (CMV) antigenemia assay for the diagnosis of CMV gastrointestinal disease (GID).

METHODS: One hundred and thirty immunocompromised patients were enrolled in this study. Patients with a history of anti-CMV treatment and who had not undergone examination using the antigenemia assay were excluded. CMV-GID was defined as the detection of large cells with intranuclear inclusions alone or associated with granular cytoplasmic inclusions by biopsy. Biopsy sections were stained with hematoxylin and eosin and immunohistochemically stained with anti-CMV. We evaluated the association between CMV-GID and patient characteristics (symptoms, underlying disease, medication, leukocyte counts, and antigenemia assay). All patients were checked with an human immunodeficiency virus (HIV) antibody test before endoscopic examination. White blood cell (WBC) counts were obtained from medical records within 1 wk of endoscopy. Leukopenia was defined as a total WBC count < 5000 cells/mm^3. For HIV patients, we also checked CD4+ counts from medical records.

RESULTS: A total of 99 patients were retrospectively selected for analysis. Of the immunocompromised patients, 19 had malignant disease, 18 had autoimmune disease, 19 had disorders of biochemical homeostasis, three had undergone transplantation, and 45 had HIV infection. A total of 50 patients had received immunosuppressive therapy. No patients had inflammatory bowel disease. Fifty-five patients were diagnosed as having CMV-GID. Univariate analysis indicated an association between HIV infection, leukopenia, and positive antigenemia and CMV-GID ($P < 0.05$). Multivariate analysis using logistic regression revealed that HIV infection and positive antigenemia were the only independent factors related to CMV-GID ($P < 0.01$). The sensitivity, specificity, positive predictive value, and negative predictive value of antigenemia for CMV-GID were 65.4%, 93.6%, 91.9%, and 71.0%, respectively. In a subgroup analy-

sis, patients with leukopenia displayed low sensitivity and high specificity. Minimal differences in accuracy were seen among patients with or without leukopenia. HIV-infected patients displayed low sensitivity and high specificity. Accuracy barely differed between HIV-positive and -negative patients. In HIV-infected patients, CD4 count < 50 cells/μL resulted in low sensitivity and high specificity. Differences in accuracy among patients were minor, regardless of CD4 count. In patients who had undergone both quantitative real-time polymerase chain reaction (PCR) and antigenemia assay, real-time PCR was slightly more accurate in terms of sensitivity than the antigenemia assay; however, this difference was not statistically significant (P = 0.312).

CONCLUSION: If the antigenemia test is positive, endoscopic lesions are acceptable for the diagnosis of CMV-GID without biopsy. The accuracy is not affected by HIV infection and leukopenia. Either PCR or the antigenemia assay are valid.

Key words: Cytomegalovirus; Gastrointestinal disease; Antigenemia assay; Real-time polymerase chain reaction; Human immunodeficiency virus infection

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INTRODUCTION

As the number of patients with immune deficiency has been increasing dramatically in recent years, the number of patients with cytomegalovirus (CMV) disease has also been increasing. CMV gastrointestinal disease (CMV-GID) frequently occurs in immunocompromised patients, particularly among those with human immunodeficiency virus (HIV) infection, transplantation, autoimmune diseases, or secondary immunodeficiency[1-8]. CMV-GID has also been described following the use of steroids, immunosuppressants, or cancer chemotherapy[1,2]. In immunocompromised patients, CMV-GID in the absence of therapy is a major cause of morbidity and mortality due to events such as massive bleeding or perforation. Therefore, diagnosis at an early stage is essential[1,2,9-12]. However, diagnosis of this infection is difficult because of wide variations in symptoms and endoscopic features depending on the infected organs[1,2].

Although the utility of various diagnostic tests for CMV-GID has been reported, the best approach is to conform the presence of CMV by histological analysis, including immunological staining by endoscopy[1-3,5,13,14]. Endoscopic examination is generally tolerated, but tissue biopsy can possibly lead to hemorrhage or perforation after endoscopic examination[10,11,15]. Endoscopists therefore hesitate to perform biopsy when deep, large, and bleeding ulcerous lesions are encountered. Patients receiving antithrombotic drugs or with thrombocytopenia also require careful consideration before biopsy.

On many occasions in recent years, noninvasive methods such as the CMV blood antigenemia assay have been applied instead of biopsy to avoid adverse effects[3,16-22]. However, few reports have examined the diagnostic value of the CMV antigenemia assay for CMV-GID, and the clinical utility of this method in immunodeficiency remains unclear[3,20-22]. Moreover, the CMV antigenemia assay requires sufficient granulocytes, and leukopenia and low CD4+ counts in patients with HIV infection could thus be expected to influence assay accuracy[3]. However, no reports have yet clarified this issue.

The aims of this study were to clarify the utility of the CMV antigenemia assay for diagnosing suspected CMV-GID, and to evaluate the accuracy of this assay under different clinical settings.

MATERIALS AND METHODS

Patient selection

One hundred and thirty immunocompromised patients with endoscopic findings who had undergone biopsy were enrolled in this study at the National Center for Global Health and Medicine (NCGM) from January 2002 to September 2009. Patients with a history of treatment with anti-CMV therapy were excluded, as were cases not examined using the CMV antigenemia assay test within 1 wk of endoscopy. Written informed consent was obtained from all patients prior to endoscopy and biopsy. All study protocols were approved by the ethics committee of NCGM.

Immunocompromised patients

Immunocompromised patients are associated with secondary immune deficiency, particularly HIV infection, hematopoietic stem cell transplantation, autoimmune diseases, malignancy, disorders of biochemical homeostasis, and use of steroids, immunosuppressants, or cancer chemotherapy.

Underlying autoimmune diseases included Rheumatoid arthritis, Systemic lupus erythematosis, Still's disease, Behcet's disease, Polymyositis, and Dermatomyositis. Diabetes mellitus, renal insufficiency/dialysis, and hepatic cirrhosis were included among the disorders of biochemical homeostasis. All patients were checked with an HIV antibody test before endoscopic examination.

Clinical manifestations

Gastrointestinal symptoms were collected from medical records written by the doctor who interviewed each per-

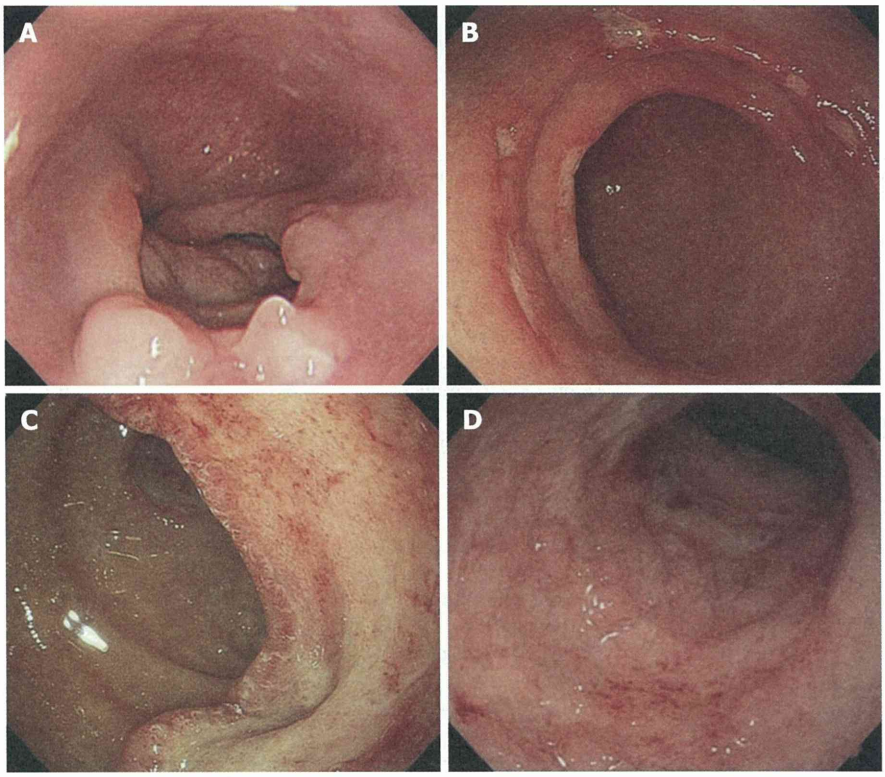

Figure 1 Endoscopic features in cytomegalovirus gastrointestinal disease. A: Deep, punched-out ulcer in the esophagus; B: Multiple, shallow ulcers in the gastric antrum; C: Large, deep ulcer in the duodenum; D: Multiple erosions and edematous mucosa with ulcer in the sigmoid colon.

son face-to-face before endoscopy. Those without records were treated as symptom free. Gastrointestinal symptoms included compromised odynophagia, epigastralgia, nausea, lower abdominal pain, diarrhea, and hematochezia. White blood cell (WBC) counts were obtained from medical records within 1 wk of endoscopy. Leukopenia was defined as a total WBC count < 5000 cells/mm^3. For HIV patients, we also checked CD4+ counts from medical records.

Antigenemia assay and quantitative real-time polymerase chain reaction

Antigenemia assay using C10/C11 monoclonal antibodies (Mitsubishi Chemical Medience, Tokyo, Japan) was performed as previously reported[16,19,20]. A positive result for the CMV antigenemia assay was defined as ≥ 1 CMV-positive cell per 150 000 granulocytes applied.

A total of 47 patients underwent additional examination with real-time polymerase chain reaction (PCR), performed basically as previously reported[3,23,24]. The minimum detection level was 200 copies/mL of plasma. A positive result for real-time CMV PCR was defined as > 200 copies/mL.

Diagnosis of CMV-GID

CMV-GID was suspected based on endoscopic findings, such as patchy erythema, edematous mucosa, multiple erosions, and ulcers (Figure 1)[25,26]. Biopsy was therefore performed when such endoscopic findings were encountered. CMV-GID was defined as the detection of large cells with intranuclear inclusions alone or associated with granular cytoplasmic inclusions by histological testing of biopsy specimens[1]. Biopsy sections were stained with hematoxylin and eosin, and immunohistochemically stained with anti-CMV (Figure 2). The results were considered positive when the above-mentioned cells showed marked brown coloration in both nuclei and cytoplasm.

Statistical analysis

We divided patients into two groups based on the presence or absence of CMV-GID. Patient characteristics and clinical findings were then compared between groups. Fisher's exact test was used to compare frequencies for patient characteristics and clinical findings, and Mann-Whitney *U* test was used for comparing age and CD4 counts. To identify clinical factors independently associated with a diagnosis of CMV-GID, stepwise logistic regression modeling was used. Sensitivity, specificity, and positive and negative predictive values of CMV antigenemia for diagnosing CMV-GID were calculated. The difference in accuracy between CMV real-time PCR and CMV antigenemia assay was compared according to the area under the curve (AUC). Values of $P < 0.05$ were considered significant. All statistical analyses were performed using Stata software (version 10, Stata Co., USA).

RESULTS

Clinical features

We excluded 10 patients who had received anti-CMV

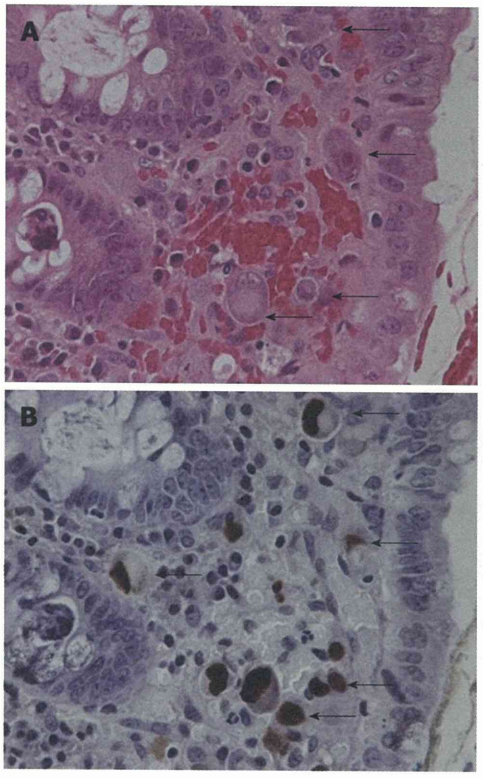

Figure 2 Pathological features in cytomegalovirus gastrointestinal disease. A: Large cells with intranuclear inclusions or associated with granular cytoplasmic inclusions (hematoxylin and eosin stain); B: Cytomegalovirus (CMV)-infected cells (arrows) show brown coloration in both nuclei and cytoplasm (immunohistochemical staining with anti-CMV).

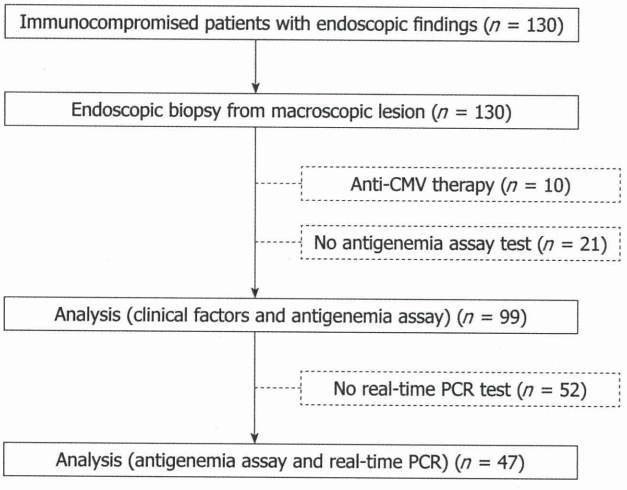

Figure 3 Study design. CMV: Cytomegalovirus; PCR: Polymerase chain reaction.

treatment, along with 21 patients who had not been examined using the CMV antigenemia assay. Thus, a total of 99 patients were retrospectively selected for analysis (Figure 3). Of the immunocompromised patients, 19 (19.1%) had malignant disease, 18 (18.1%) had autoimmune disease, 19 (19.1%) had disorders of biochemical homeostasis, three (3%) had undergone transplantation, and 45 (45.5%) had HIV infection. A total of 50 patients (50.1%) had received immunosuppressive therapy. No

Table 1 Clinical factors for cytomegalovirus gastrointestinal disease (univariate analysis)

	CMV-GID (*n* = 52)	Non-CMV-GID (*n* = 47)	*P*-value
Age (yr, mean ± SD)	46.8 ± 16.2	56.6 ± 17.8	0.050
Male sex	30	41	0.098
Immunodeficiency disease			
HIV infection	33	12	< 0.001
Malignancy	9	10	0.617
Solid cancer	1	3	
Hematological cancer	8	7	
Autoimmune disease	7	11	0.200
Disorders of biochemical homeostasis	8	11	0.312
Chronic renal failure	1	2	
Liver cirrhosis	0	2	
Diabetes mellitus	7	7	
Transplantation	1	2	
Immunosuppressive therapy	25	25	0.611
Steroids	22	19	
Immunosuppressants	8	4	
Chemotherapy	4	4	
Positive CMV antigenemia	34	3	< 0.001
Leukopenia	35	21	0.023
With gastrointestinal symptoms	34	34	0.456

HIV: Human immunodeficiency virus; CMV: Cytomegalovirus; GID: Gastrointestinal disease.

patients had inflammatory bowel disease (IBD). Fifty-five patients were histologically diagnosed with CMV-GID. Univariate analysis (Table 1) identified HIV infection ($P < 0.001$), leukopenia ($P = 0.023$), and positive CMV antigenemia assay ($P < 0.001$) as being associated with CMV-GID. Multivariate analysis revealed HIV infection [odds ratio (OR), 6.57; 95% CI: 2.1-20.2, $P = 0.001$] and positive CMV antigenemia assay (OR, 33.3; 95% CI: 8.1-136.2, $P < 0.001$) as the only factors independently correlated with CMV-GID.

HIV-infected patients included 44 men (97.8%) and their mean age was 42.1 years (range, 25-74 years). Median CD4 count was 57 (interquartile range, 17-111). Patients with CMV-GID showed significantly lower CD4 counts than those without CMV-GID (median CD4 count; CMV-GID *vs* non-CMV-GID: 24 *vs* 150, $P < 0.001$).

Accuracy of CMV antigenemia assay for diagnosing CMV-GID

A positive CMV antigenemia assay showed low sensitivity and high specificity (Table 2). In a subgroup analysis, patients with leukopenia displayed low sensitivity and high specificity. Minimal differences in accuracy were seen among patients with or without leukopenia. HIV-infected patients displayed low sensitivity and high specificity. Accuracy barely differed between HIV-positive and -negative patients. In HIV-infected patients, CD4 count < 50 cells/μL resulted in low sensitivity and high specificity. Differences in accuracy among patients were minor, regardless of CD4 count.

In patients who had undergone both quantitative real-

Table 2 Diagnostic accuracy of cytomegalovirus antigenemia for detecting cytomegalovirus gastrointestinal disease

Subgroups	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
All patients (n = 99)	65.40% (55.4-74.9)	93.60% (87.3-97.7)	91.90% (84.7-96.4)	71.00% (60.7-79.4)
Patients with leukopenia (n = 56)	68.60% (54.0-79.7)	100% (93.6-100)	100% (93.6-100)	65.60% (52.2-78.2)
Patients without leukopenia (n = 43)	58.80% (42.1-73.0)	88.50% (74.9-96.1)	76.90% (61.4-88.2)	76.70% (61.4-88.2)
HIV-infected patients (n = 45)	63.60% (48.8-78.1)	100% (92.2-100)	100% (92.2-100)	50.00% (35.8-66.3)
Non-HIV-infected patients (n = 54)	68.40% (54.5-80.5)	91.40% (79.7-96.9)	81.30% (68.6-90.7)	84.20% (70.7-92.1)
HIV-infected patients with CD4 count < 50 (n = 22)	61.90% (40.7-82.8)	100% (84.6-100)	100% (84.6-100)	11.10% (1.12-29.2)
HIV-infected patients with CD4 count ≥ 50 (n = 23)	66.70% (42.7-83.6)	100% (85.2-100)	100% (85.2-100)	73.30% (51.6-89.8)

HIV: Human immunodeficiency virus; PPV: Positive predictive value; NPV: Negative predictive value.

Table 3 Comparison of diagnostic accuracy for detecting cytomegalovirus gastrointestinal disease between antigenemia assay and quantitative real-time polymerase chain reaction (n = 47)

	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
CMV real-time PCR	73.00% (57.4-84.4)	100% (92.5-100)	100% (92.5-100)	50.00% (36.1-65.9)
CMV antigenemia assay	64.90% (50.7-79.1)	100% (92.5-100)	100% (92.5-100)	43.50% (28.3-57.8)

CMV: Cytomegalovirus; PPV: Positive predictive value; NPV: Negative predictive value; PCR: Polymerase chain reaction.

time PCR and antigenemia assay (Table 3), real-time PCR was slightly more accurate in terms of sensitivity than the antigenemia assay; however, this difference was not statistically significant (P = 0.312).

DISCUSSION

CMV-GID is a major cause of morbidity and mortality in immunocompromised patients; therefore, diagnosis at an early stage is essential[1,2,5,8,9]. However, clinical diagnosis of this disease can be difficult, as physicians need to consider various underlying diseases and clinical presentations. Patients at high risk of CMV-GID have been reported as those with HIV infection or undergoing steroid therapy or cancer therapy[1]. The present study identified HIV infection as one of the independent factors in secondary immunodeficiency diseases. This is because the number of eligible subjects was small and included immunocompromised patients while excluding immunocompetent patients.

Among the various clinical manifestations, a positive CMV antigenemia assay was found to be a useful factor for diagnosing CMV-GID. The CMV antigenemia assay is one of the most widely used methods for detecting reactivation of CMV infection, but few studies have examined the diagnostic value for CMV-GID[3,21,22]. Our findings demonstrated 65% sensitivity and 94% specificity of the CMV antigenemia assay for diagnosing CMV-GID. Mori *et al*[3] reported that only four of 19 patients (21%) developed a positive CMV antigenemia assay before developing CMV-GID; however, all 19 patients subsequently tested positive for CMV antigenemia after diagnosis of CMV-GID. There is a possibility that patients with CMV-GID will develop a positive CMV antigenemia assay at follow-up, but our study did not assess this process after diagnosis of CMV-GID. Fica *et al*[21] also reported that the CMV antigenemia assay result was positive for 18 of 31 patients (58%) with CMV end-organ disease, with CMV-GID (71%) as the most frequent cause. However, these studies were limited in that the number of subjects was small and the specificity of the CMV antigenemia assay was unknown. Jang *et al*[22] recently reported that the sensitivity and specificity of the CMV antigenemia assay for diagnosing CMV-GID were 54% and 88%, respectively, in patients with secondary immunodeficiency disease. The reports mentioned above showed that the CMV antigenemia assay has low sensitivity for the diagnosis of CMV-GID, which is consistent with our results.

It has been reported that sufficient granulocytes are essential in evaluating CMV using the antigenemia assay. Previous studies using the antigenemia assay to diagnose CMV-GID have reported that most of the patients were transplant recipients and were mostly HIV-negative[3,21,22]. No studies have compared the assay among groups of HIV-positive/-negative patients and among groups with or without leukopenia. In patients with HIV infection, most cases of CMV-GID have known to occur with CD4 counts < 50 cells/μL[2,4]. However, whether the accuracy of the antigenemia assay is affected by the immunosuppressed state has not been elucidated. We suspected that such different groups would show differences in the accuracy of CMV antigenemia assay, but found little difference. This suggests that our results are applicable to these different groups in clinical practice.

Besides the CMV antigenemia assay, quantitative real-time PCR is also used for detecting reactivation of CMV infection, and is considered more useful for predicting CMV disease than the CMV antigenemia assay[23,24]. In our study, quantitative real-time PCR and CMV antigenemia assay were performed simultaneously on 47 patients. The PCR method showed a tendency toward slightly higher sensitivity, but no significant differences were evident. In Japan, the CMV PCR method has not been widely used in

clinical practice because of the higher costs compared to the antigenemia assay. We thus do not recommend use of PCR methods in the sub-diagnosis of CMV-GID, as the antigenemia assay is just as valid.

One limitation of this study was the single-center, retrospective nature of the investigation. A significant difference might not have been confirmed among independent factors due to the small number of patients. Further studies of more patients are needed. Another limitation is the verification bias, which is dependent on the physician's decision to perform the antigenemia assay.

The diagnosis of CMV-GID is considered as the gold standard for identifying CMV cells in tissue samples from endoscopic biopsy[1,2,13]. Various endoscopic findings are present in CMV-GID, such as ulcer and mucosal inflammation[25,26]; however, physicians may not perform a biopsy in cases only showing mucosal inflammation without ulcer. Even in cases of severe ulceration that is deep or bleeding, physicians may hesitate to perform a biopsy. In such cases, a diagnosis of CMV-GID may not be reached. Our results suggest that the CMV antigenemia assay is useful for the sub-diagnosis of CMV-GID in immunocompromised patients with endoscopic findings. Considering the high specificity of the test, the use of this method before endoscopy could potentially avoid complications due to biopsy. Positive antigenemia is also useful for evaluating improvements in CMV-GID after anti-CMV treatment. However, the low sensitivity means that if the antigenemia assay yields negative results, biopsy and immunohistochemical staining of specimens with anti-CMV will be required for diagnosis. Negative antigenemia assay results may require a repeat examination at a different time[3]. Moreover, the use of different non-invasive methods such as quantitative PCR should be considered.

In conclusion, the CMV antigenemia assay is highly useful for diagnosing CMV-GID. If the antigenemia assay provides positive results, the presence of endoscopic lesions should allow diagnosis of CMV-GID without biopsy. The accuracy of the test is unaffected by the presence of HIV infection or leukopenia.

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COMMENTS

Background

Cytomegalovirus (CMV) gastrointestinal disease (GID) is a major cause of morbidity and mortality in immunocompromised patients; therefore, diagnosis at an early stage is essential. However, clinical diagnosis of this disease can be difficult, as physicians need to consider various underlying diseases and clinical presentations.

Research frontiers

The diagnosis of CMV-GID requires an endoscopic biopsy, which is invasive and may lead to complications. While the CMV antigenemia assay is one of the most widely used methods for detecting reactivation of CMV infection, few studies have examined its diagnostic value for CMV-GID. In this study, the authors demonstrate that the CMV antigenemia assay was highly useful for diagnosing CMV-GID.

Innovations and breakthroughs

There were no studies of diagnosis on CMV-GID related factors using multivariate analysis. In this study, among the various clinical manifestations, human immunodeficiency virus (HIV) infection and positive CMV antigenemia assay were found to be a useful factors for diagnosing CMV-GID by multivariate analysis. As for accuracy of CMV antigenemia for diagnosing CMV-GID, recent reports have highlighted that the sensitivity and specificity were 54% and 88%, respectively, in patients with secondary immunodeficiency disease. However, no studies have compared the assay among groups of HIV-positive/-negative patients and among groups with or without leukopenia. In this study, the sensitivity, specificity, positive predictive value, and negative predictive value of antigenemia for CMV-GID were 65.4%, 93.6%, 91.9%, and 71.0%, respectively. In addition, its accuracy was not affected by the presence of HIV infection and leukopenia. These results are very useful for diagnosing CMV-GID by clinical physicians.

Applications

Considering the high specificity of the test, use of this method before endoscopy could potentially avoid complications due to biopsy. However, the low sensitivity means that if the antigenemia assay yields negative results, biopsy and immunohistochemical staining of specimens with anti-CMV will be required for diagnosis. Negative antigenemia assay results may require repeat examination at a different time. Moreover, the use of different non-invasive methods such as quantitative polymerase chain reaction should be considered.

Peer review

This paper is interesting and it could be valuable for other researchers.

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Living Donor Liver Transplantations in HIV- and Hepatitis C Virus-Coinfected Hemophiliacs: Experience in a Single Center

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Background. Although almost all human immunodeficiency virus (HIV)-infected Japanese hemophiliacs are coinfected with hepatitis C virus (HCV), the outcome of living donor liver transplantation (LDLT) in such patients in terms of survival rate, perioperative complications, and recovery of coagulation activity is poorly understood.
Patients and Methods. Six HIV-positive hemophiliacs underwent LDLT for HCV-associated advanced cirrhosis. The mean CD4 T-cell count at transplantation was 376±227/μL.
Results. The 1-, 3-, and 5-year survival rates were 66%, 66%, and 50%, respectively. Fatal perioperative bleeding related to hemophilia was not observed. Two patients died within 6 months after transplantation due to graft failure. HIV infection was well controlled in all patients who survived longer than 6 months. Two patients (genotype 2a and 2+3a) achieved a sustained viral response and both of them were alive at the end of follow-up period, whereas one patient (genotype 1a+1b) died of decompensated cirrhosis 4 years after transplantation due to recurrent HCV infection.
Conclusions. HIV/HCV-coinfected hemophiliacs can safely undergo LDLT. Hemophilia was clinically cured after successful transplantation. A good outcome can be expected as long as postoperative hepatitis C is controlled with interferon/ribavirin combination therapy.

Keywords: Hepatitis C virus, Living donor liver transplantation, HIV, HAART.

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Because of the availability of highly active antiretroviral therapy (HAART), the life expectancy of patients infected with human immunodeficiency virus (HIV) has dramatically improved (*1*). Death from opportunistic infections has decreased and, as the result, non-acquired immune deficiency syndrome (AIDS)-defining complications such as hepatic diseases, cardiovascular diseases, or non-AIDS malignancies have emerged as the most important problems (*2, 3*).

Hepatitis C virus (HCV) and HIV often coinfect due to their shared route of transmission. A recent report indicated that approximately 20% of HIV-infected people in Japan are coinfected with HCV (*4*), a large proportion of whom are hemophiliacs. Approximately 1500 hemophiliacs were infected with HIV through non heat-treated concentrated coagulation factor administration between 1981 and 1985, and 98% of them were also infected with HCV. The coexistence of HIV infection with HCV accelerates the progression of liver fibrosis (*5*) and attenuates the efficacy of interferon (IFN) treatment for HCV (*6, 7*). A considerable number of such coinfected patients suffer from decompensated cirrhosis or hepatocellular carcinoma (HCC) (*8*). In the HAART era, AIDS-related death is gradually decreasing (*9*) and HCV-

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related liver diseases have become the leading cause of death in Japanese hemophiliacs (*10*).

The only curative treatment for end-stage liver disease is liver transplantation. In the pre-HAART era, HIV infection was considered an absolute or relative contraindication for transplantation. Several cases were reported during that period (*11*, *12*), but the outcomes were not always satisfactory. In the HAART era, more than 50 cases of HIV-positive liver transplantation have been reported (*13–21*), and survival after liver transplantation seems to be more promising.

The absolute number of deceased donor livers in Japan is small, and living donor liver transplantation (LDLT) is the mainstay of liver transplantation. We reported the first LDLT in an HIV-positive hemophiliac in 2002 (*22*). Here, we present a series of six cases of LDLT in HIV/HCV-coinfected hemophiliacs performed at the University of Tokyo Hospital between 2001 and 2004.

RESULTS

Survival

The 1-, 3-, and 5-year survival rates were 66%, 66%, and 50%, respectively. Two patients (cases 2 and 5) died on postoperative day (POD) 99 and 156, respectively. The causes of early death were graft failure and bleeding from cytomegalovirus (CMV) enteritis (case 2) and graft failure suspected to be cholestatic hepatitis (case 5). One patient died 50 months after LDLT due to recurrent HCV-related cirrhosis.

Results of Antiviral Therapy for Recurrent Hepatitis C in the Graft

After LDLT, all but one (case 2) patients received combination therapy with IFN (standard or pegylated form) and ribavirin. Case 3 was treated for biopsy-proven recurrent hepatitis C, whereas the other four were treated preemptively (started on POD, 10–70 days). Duration of anti-HCV therapy was 12 months in case early viral response was achieved. Cases 1 and 3 achieved sustained viral response (SVR). Case 3 suffered from HCV-related cholestatic hepatitis on POD 38, which responded well to combination therapy with IFN and ribavirin and he eventually achieved SVR. The other patients did not achieve SVR. Cases 4 and 6 showed a biochemical response and were on maintenance antiviral therapy. In case 6, tacrolimus was switched to cyclosporine A 15 months after LDLT to suppress HCV replication. This lead to a transient 10-fold decrease in HCV-RNA, but it returned to the previous value within several months.

Results of Antiretroviral Therapy After LDLT

Antiretroviral therapy was transiently terminated during the perioperative period. The timing of reintroduction was individualized according to the CD4 count, HIV viral load, general status such as surgical complication and the result of liver function tests. One patient (case 1) has continued to maintain a high CD4 count without antiretroviral therapy. One patient (case 2) died before antiretroviral reintroduction.

The remaining four patients started antiretroviral therapy at a median of 56.5 days after LDLT (range, 43–485 days). The choice of the antiretroviral drug was individualized according to each patient's antiretroviral history and accumulated resistance mutations. A protease inhibitor-based combination was selected in all cases. All but one patient (case 5) tolerated antiretroviral therapy and had an excellent response. The blood concentration of the immunosuppressant increased drastically from the first day of protease inhibitor administration, which was controlled by close monitoring and dosage modification.

Elevation of serum alkaline-phosphatase and gamma-glutanyl-transpeptidase values was observed in all patients after antiretroviral reintroduction. Other significant adverse effects include severe allergic reaction to lamivudine (case 3) and liver failure, which was clinically diagnosed to be cholestatic hepatitis as an immune reconstitution inflammatory syndrome against HCV (case 5).

One patient (case 3) developed Burkitt leukemia 38 months after LDLT. His CD4 count at that time was 480/μL and HIV-RNA was undetectable. Combination chemotherapy using cyclophosphamide, vincristine, doxorubicin, and dexamethasone (*23*) was effective, and he eventually achieved complete remission. Other opportunistic infections included multiple abscess formation at the surgical site in two patients (case 2 by methicillin-resistant *Staphylococcus aureus* and case 5 by multi-drug resistant *Pseudomonas aeruginosa*). Positive CMV antigenemia was observed in all cases. However, only one patient (case 2) presented with clinically overt organ damage.

Restoration of Coagulation After LDLT

Except for case 5, replacement became unnecessary within 1 week after operation. In case 5, in addition to insufficient endogenous coagulation factor production, re-operation was necessary several times, and the coagulation factor replacement could not be withdrawn. Cases 2 and 6 again required coagulation factor replacement after graft failure became apparent.

Outcome of the Donors

All donors were alive without major complications at the point of analysis. Two donors were considered obligate carriers of hemophilia and one of them (donor of case 5) showed relatively low coagulation activity, but none of the donors experienced abnormal bleeding requiring coagulation factor administration. The donor of case 5 experienced transient decrease in factor IX activity after liver resection. However, the value of coagulation activity recovered without supplementation.

DISCUSSION

Recurrence of hepatitis C is the most important problem in treating HCV-positive hemophiliac patients. Recent reports indicate that HIV/HCV-coinfected liver recipients have a relatively lower survival rate than HCV-monoinfected liver recipients, although the difference is not significant. In our series, two of three deaths were related to recurrent HCV, and two patients experienced fibrosing cholestatic hepatitis. Cholestatic hepatitis is characterized by a high rate of HCV replication and a paucity of inflammatory activity, and the risk might increase in LDLT recipients (*24*, *25*). In our center, IFN therapy is usually introduced preemptively as soon as possible. In our series, two cases infected with non-1b virus achieved SVR, whereas others did not achieve SVR. A report demonstrated the effectiveness of maintenance therapy with

pegylated (PEG)-IFN plus ribavirin (*26*), but this efficacy was not apparent in our series. Combination antiviral therapy with protease and polymerase inhibitors may improve the treatment results in the future.

With regard to HIV infection, when to restart antiretroviral therapy after LDLT has remained a question. Hemophiliacs often have a long-term treatment history. Five of six cases had a multiple history of treatment failure, and as a result, only one or two reliable antiretroviral combinations were available to each patient in that era. Protease inhibitors, key drugs for successful HIV suppression in such cases have a potential risk of liver toxicity, especially in those with HCV coinfection (*27*). Unlike whole liver transplantation, the initial graft size is relatively small in LDLT. The graft gradually increases its volume within several weeks after transplantation, and an unfavorable effect of antiretroviral treatment on graft growth during this period is a concern. Moreover, unintended treatment interruption due to early phase complications may result in further accumulation of resistance-associated mutations. Taking these issues into account, we delayed starting antiretroviral therapy until at least 4 weeks after LDLT. It is obvious, however, that earlier antiretroviral reintroduction has more benefit toward reducing opportunistic infections and improving the result of anti-HCV therapy after LDLT. The effectiveness and safety of a new class antiretrovirals, raltegravir (*28*), and enfuvirtide (*29*), were recently reported, and these compounds may play an important role in the management of HIV-infected split-graft recipients.

In our series, the immunosuppressant trough level was targeted to the same level as that in HIV-negative cases. It is not known, however, whether HIV-infected patients, particularly those with a relatively lower CD4 cell count, need the same blood level of immunosuppressants. Moreover, the CD4 cell count, may not act as accurate surrogate marker for immune function in those taking an immunosuppressant or steroid. In case 2, recurrent bleeding from CMV intestinal ulcer eventually led to death after immunosuppression was intensified to treat severe graft rejection. In this case, antiretroviral therapy could not be reintroduced because of severe liver damage, which might enhance excess immunosuppression. A more precise indicator than CD4 count and immunosuppressant level is needed. Dose modification of immunosuppressive drugs using an immune function assay (*30*) may contribute to more precise management, especially in HIV-coinfected patients.

A considerable number of HIV/HCV-coinfected patients are suffering from decompensated cirrhosis or HCC (*8*), and some of them are potential candidates for future liver transplantation. The shortage of deceased donor liver grafts is a major problem worldwide. LDLT can overcome such a problem. Clearly, regenerative medicine will have an important role in this field in the future. Those patients who are already in a cirrhotic state, however, cannot wait for such an innovative modality to be established. In our series, all patients who tolerated antiretroviral therapy achieved good HIV control, and those who cleared HCV survived long. Clinical cure of hemophilia after successful transplantation drastically improved the patients' quality of life. Cure of hemophilia also lead to considerable cost reduction. LDLT continues to have an important role in HIV-infected hemophiliacs.

MATERIALS AND METHODS

From April 2001 to October 2004, nine HIV/HCV-coinfected patients were referred to the University of Tokyo hospital for LDLT. The indication was HCV-related end-stage liver disease.

HIV-positive patients should meet the same standard criteria for liver transplantation as HIV-negative patients. The criteria for accepting candidates for LDLT were absolute CD4 T lymphocyte count more than 200/μL, or more than 14% CD4 proportion to total lymphocytes when hypersplenism-related leukocytopenia was considered the cause of an apparent decrease in the CD4 count. Undetectable HIV RNA was not required as long as effective HIV suppression was expected after transplantation. Exclusion criteria related to HIV infection were active AIDS-defining diseases except for esophageal candidiasis. All cases were approved by the ethics committee at the University of Tokyo. Donor was selected from those with spontaneous will and within the third-degree consanguinity of the patient. Those with abnormal coagulation values were excluded from candidate for the donor.

Two patients did not meet the criteria (one with concomitant uncontrollable fungal infection and one without appropriate donor). One patient retracted consent before operation. Finally, six HIV/HCV-coinfected hemophiliacs underwent LDLT. Two patients were transplanted emergently (within 2 weeks after referral) because of progressive hepatic encephalopathy and hepatorenal syndrome. None of the patients had concomitant active hepatitis B, HCC, or other malignancies. The patient characteristics are summarized in Table 1.

The appropriate type of concentrated coagulation factor was administered during the perioperative period. Concentrated coagulation factor was administered as a bolus just before the operation to achieve 100% coagulating

TABLE 1. Patient characteristics at LDLT and outcome

Case	Age/ sex	Type of hemophilia	HCV genotype	HCV-RNA at LDLT (KIU/mL)	HIV load (copy/mL)	MELD at LDLT	CCr	HTN/ DM	BMI	Graft	Graft size (%SLV)	ACR	CMV	Survival (mo)	Donor
1	41M	B	2a	3	UD	23	24	N/N	19.1	Right	66	0	1	Alive (115)	Brother
2	28M	A	2a, 2b	1410	6.2×10^4	15	76	N/N	23.4	Right	57	2	2	Died (3)	Mother
3	30M	A	1b, 3a	740	3.2×10^4	15	78	N/N	21.5	Right	42	1	2	Alive (96)	Mother
4	38M	A	1b, 3a	200	UD	34	69	N/N	20.0	Right	47	1	1	Alive (82)	Sister
5	31M	B	1a	747	2.6×10^4	18	72	N/N	24.3	Right	47	2	3	Died (5)	Mother
6	32M	B	1a, 1b	41	UD	48	62	N/N	25.2	Right	63	0	0	Died (50)	Father

HCV, hepatitis C virus; LDLT, living donor liver transplantation; HIV, human immunodeficiency virus; MELD, model for end-stage liver disease; CCr, creatine clearance; HTN, hypertension; DM, diabetes mellitus; BMI, body mass index; SLV, standard liver volume; ACR, acute cellular rejection; CMV, cytomegalovirus; UD, undetectable.

factor activity, followed by continuous infusion to maintain greater than 80% activity during the operation. Fresh-frozen plasma was also replaced. Initial dosage of the coagulation factor was calculated based on the results of preoperative pharmacokinetic studies, and the rate of continuous infusion was adjusted as necessary by periodical monitoring of coagulation factor activity.

Tacrolimus and steroids based immunosuppression was planned as previously described (*31*). The target tacrolimus trough level was same as that for the HIV-negative population. Moderate to severe rejection was treated with pulse steroids±mycophenolate mofetil.

The preoperative HCV-RNA value was positive in all subjects. The HCV genotype is listed in Table 1. All patients underwent concomitant splenectomy (*32*). Preemptive anti-HCV therapy with IFN (standard or pegylated form) plus ribavirin was planned after LDLT (*33*). Postoperative CMV reactivation was monitored using a pp65 antigen detecting method (CMV antigenemia), and a positive result was preemptively treated with ganciclovir (*34*) or valganciclovir.

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IL-6 levels have been assayed using enzyme-linked immunosorbent assays (ELISAs), whereas Haddow *et al.* [4] used a multiplex bead array assay (MBAA). Plasma IL-6 levels in HIV patients may differ significantly when assayed by ELISA or MBAA [9].

It is now clear that assays of selected biomarkers can assist in both the prediction and diagnosis of TB-IRIS and ART-TB. Large-scale studies are now needed to delineate the value of these assays as diagnostic tests. In doing this, consideration should be given to the use of both plasma and IGRA plasma from antigen-stimulated and antigen-unstimulated tubes and also to the method used to assay cytokines and chemokines.

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Conflicts of interest

There are no conflicts of interest.

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Antiretroviral therapy alone resulted in successful resolution of large idiopathic esophageal ulcers in a patient with acute retroviral syndrome

Esophageal ulceration is a well known complication of end-stage HIV infection, and also patients with acute retroviral syndrome [1–5]. Various infectious agents can cause esophageal diseases, including *Candida* species, cytomegalovirus (CMV), and herpes simplex virus (HSV). However, in a large proportion of patients with esophageal ulcers, no agent can be identified and ulceration is considered idiopathic [1,2]. Here, we report a case of an idiopathic esophageal ulcer with concomitant primary HIV-1 infection.

A previously healthy 36-year-old Japanese male homosexual presented with odynophagia, fever, and headache. Five days after the onset, he was diagnosed with oral candidiasis and treated with fluconazole for 5 days. Despite the treatment, odynophagia did not improve. The result of voluntary HIV screening 1 month before the onset of the symptoms was negative. However, 8 days after the initial presentation, ELISA for anti-HIV antibody was positive. The patient was referred to our hospital 2 weeks after the onset of odynophagia.

On admission, the patient was alert and oriented with body temperature of 38.5°C. Physical examination showed no oral candidiasis, peripheral lymphadenopathy, or rashes. The patient could not swallow any solid food due to the severe odynophagia. Laboratory tests at admission showed elevated liver enzymes (aspartate aminotransferase, 335 IU/l; alanine aminotransferase, 357 IU/l) and elevated lactate dehydrogenase (1182 IU/l). Cerebrospinal fluid analysis showed almost normal findings [leukocyte count, 5.9 cells/μl (61% mononuclear cells); glucose level, 52 mg/dl (serum glucose, 100 mg/dl); and protein, 29 mg/dl]. Gram and India ink staining were negative, as were bacterial and mycobacterial cultures and the cryptococcal antigen test. Serum immunoglobulin

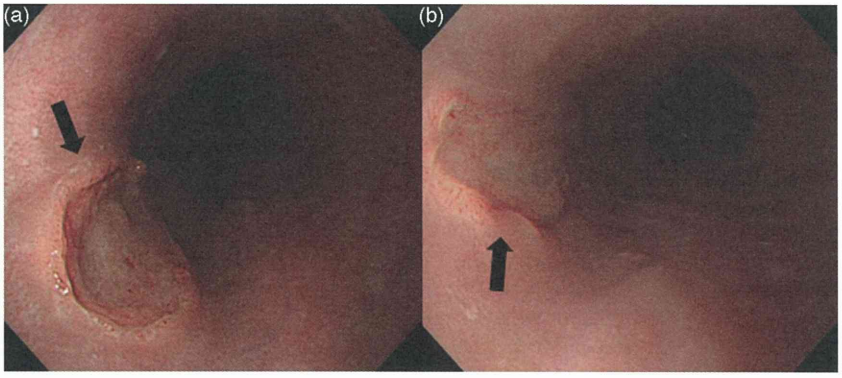

Fig. 1. Endoscopic appearance of idiopathic esophageal ulcer. Before antiretroviral therapy (a) and on the fifth day of antiretroviral therapy (b). The black arrow indicates the esophageal ulcer.

(IgG) and IgM antibodies for CMV were positive, and IgG was positive and IgM was negative for HSV. Whole blood PCR was negative for both CMV and HSV.

Western blotting for HIV antibodies revealed positivity only for GP41. Plasma HIV-1 RNA viral load was 2 200 000 copies/ml. The $CD4^+$ and $CD8^+$ cell counts were 140 and 1057 cells/μl, respectively.

Endoscopy on day 3 identified five large, discrete, and well circumscribed esophageal ulcers, with the largest measuring 15 mm in diameter Fig. 1a. There was no evidence of candidiasis. Three biopsies were obtained from the ulcers and histopathology revealed nonspecific findings: infiltration by numerous neutrophils into the squamous epithelium and lamina propria. No intranuclear or intracytoplasmic inclusion bodies were found. Immunohistochemical staining for CMV and HSV was negative.

Antiretroviral therapy of lopinavir/ritonavir with tenofovir/emtricitabine was initiated on the fourth day of admission. The following day, the patient became afebrile, and 2 days later the odynophagia and headache disappeared. Endoscopy on day 5 of antiretroviral therapy demonstrated substantial reduction in the size and depth of esophageal ulcers (Fig. 1b). The patient was discharged on day 13. One month after discharge, all bands of western blot assay turned positive, confirming the diagnosis of acute retroviral syndrome. Plasma HIV-1 RNA became undetectable 3 months after initiation of antiretroviral therapy.

To our knowledge, this is the first report demonstrating the resolution of idiopathic esophageal ulcers on antiretroviral therapy alone in a patient with untreated HIV infection. Steroids are the standard treatment of idiopathic esophageal ulcers [2,6]. However, due to their immunosuppressive effects and various other adverse effects, they are not generally recommended in immunocompromised patients. The cause of idiopathic esophageal ulcer is considered to include HIV-associated T-cell activation, which induces apoptosis of esophageal mucosa [5,7]. This probable cause supports the rationale of using antiretroviral therapy for idiopathic esophageal ulcer.

The diagnosis of idiopathic esophageal ulcer is accomplished by excluding other infectious agents known to cause esophageal ulceration, notably CMV and HSV, by histopathological and immunological examinations of endoscopically obtained specimens [2,8]. Pill-induced esophagitis and gastroesophageal reflux disease need to be excluded [2]. The presented case was positive for anti-CMV IgM antibody, suggestive of primary infection or reactivation of CMV. It is, thus, difficult to completely exclude CMV-related esophageal ulceration in this patient. However, histopathology showed no evidence of CMV-related cytopathic changes, and immunohistochemical staining for CMV was also negative. Furthermore, initiation of antiretroviral therapy resulted in rapid healing of the esophageal ulcers, suggesting that primary HIV infection was the cause of ulceration. It is noteworthy that anti-CMV IgM antibody could become falsely positive during acute infection with other viruses presumably because of shared epitopes among other antigens [9,10]. Thus, idiopathic esophageal ulcer is the most probable diagnosis in this case.

It is concluded that in untreated patients with HIV lacking identifiable infectious agents, such as CMV or HSV, in endoscopic biopsies from esophageal ulcer, antiretroviral therapy alone could produce a favorable outcome.

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