Table 1. Characteristics of All Patients Who Underwent Anti-*E. histolytica* Testing (n = 1303)

	Anti-*E. histolytica* Negatives (n = 1026)	Anti-*E. histolytica* Positives (n = 277)	*P* Value
Age,years (range)	36 (18–77)	37 (19–74)	.06
Japanese nationality, no. (%)	921 (89.8%)	250 (90.3%)	.81
Male sex, no. (%)	960 (93.6%)	272 (98.2%)	.003
MSM, no. (%)	789 (76.9%)	245 (88.4%)	<.001
TPHA test positive, no. (%)	366/1012 (36.2%)	151/275 (54.9%)	<.001
HBV exposure,[a] no. (%)	524/1017 (51.5%)	187/272 (68.8%)	<.001
HCV Ab positive, no. (%)	40/1011 (4.0%)	5/273 (1.8%)	.09
Past history of IA, no. (%)	13 (1.3%)	60 (21.7%)	<.001
Diagnosis of IA at first visit, no. (%)	1 (0.1%)	7 (2.5%)	<.001

Abbreviations: Ab, antibody; Anti-*E. histolytica*, anti *Entamoeba histolytica* antibody; HBV, hepatitis B virus; HCV, hepatitis C virus; IA, invasive amebiasis; MSM, men who have sex with men; TPHA, *Treponema pallidum* hemagglutination.

[a] HBV exposure: HBsAg-positive or HBsAb-positive, and/or HBc-Ab positive.

such as sexuality and serology status of other STIs, was estimated with univariate Cox proportional hazards regression. We also conducted multivariate Cox hazards regression analysis using variables identified in univariate analysis with *P* values of < .20. In all analyses, statistical significance was defined as 2-sided *P* value of < .05. We used the hazard ratio (HR) and 95% confidence interval (95%CI) to estimate the impact of each variable on the development of invasive amebiasis. All statistical analyses were performed using the Statistical Package for Social Sciences (SPSS Inc., Chicago, IL).

RESULTS

Clinical Characteristics of Asymptomatic Anti-*E. histolytica*-positive HIV-1-infected Patients

A total of 1519 patients were referred to our hospital during the study period. Anti-*E. histolytica* testing was conducted in 1303 patients at first visit, including 73 with history of invasive amebiasis, and anti-*E. histolytica* was positive in 277 of these (21.3%). Among the anti-*E. histolytica*-positive individuals, the rates of MSM (88.4%) and those with previous exposure to syphilis (TPHA test positive) (54.9%) and HBV (68.8%) were higher than those of anti-*E. histolytica*-negatives individuals, indicating that sexually active MSM are prone to *E. histolytica* infection among HIV-1-infected individuals in Japan (Table 1). Eight patients were diagnosed with invasive amebiasis at first visit, including 7 cases of amebic colitis and 1 case of amebic liver abscess, and they were treated immediately with metronidazole.

Incidence of Invasive Amebiasis During Follow-up of HIV-1 Infected Individuals

To assess the frequency of development of invasive amebiasis in patients free of symptomatic invasive amebiasis and who had not previously received nitroimidazole therapy, we excluded 96 patients from the analysis, including 73 patients because they had been treated previously for invasive amebiasis, and 23 patients (7 cases of amebic colitis, 1 case of amebic liver abscess, and 15 asymptomatic but anti-*E. histolytica*-positive cases treated preemptively) because they were treated with nitroimidazole at first visit (Figure 1). The remaining 1207 patients, including 195 anti-*E. histolytica*-positive patients (16.2%), were followed-up for median period of 25.3 months (interquartile range: 7.0–47.2). During the follow-up period, 18 patients developed invasive amebiasis (median time to onset: 9.1 months), including amebic appendicitis in 1 patient

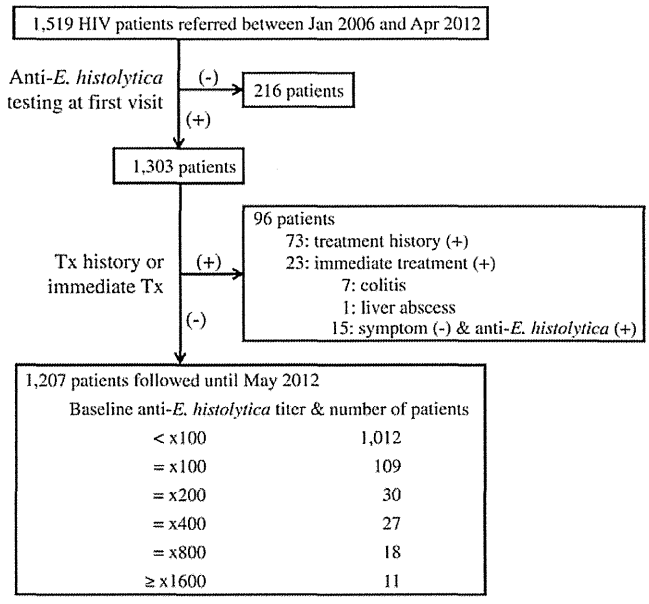

Figure 1. Flow diagram of patient recruitment process. Abbreviations: Anti-*E. histolytica*, anti-*Entamoeba histolytica* antibody; IA, invasive amebiasis; Tx, treatment.

Table 2. Comparison of Clinical Characteristics of Patients With and Without Invasive Amebiasis

	Amebic Colitis (n = 11)	Extraintestinal IA[a] (n = 7)	Non-IA (n = 1189)	*P* Value IA vs Non-IA
Age (years), average (SD)	35.9 (12.3)	38.2 (11.0)	37.5 (10.8)	.81
Japanese nationality, no. (%)	10 (90.9)	6 (85.7)	1068 (89.8)	.71
Male sex, no. (%)	11 (100)	7 (100)	1119 (94.1)	.62
MSM, no. (%)	11 (100)	6 (85.7)	929 (78.1)	.15
TPHA test-positive, no. (%)	5 (45.5)	2 (28.6)	451/1175 (38.4)	.91
HBV exposure,[a] no. (%)	6 (54.5)	5 (71.4)	630/1178 (53.5)	.15
HCV Ab-positive, no. (%)	0/11 (0)	0/7 (0)	42/1172 (3.6)	1.00
Anti-*E. histolytica* at baseline, median (IQR)	×100 (<×100–×800)	×400 (×100–×400)	<×100 (<×100–<×100)	<.001
Anti-*E. histolytica* at the onset of IA, median (IQR)	×800 (×200–×800)	×400 (×100–×800)	. . .	
Follow-up period, median months (IQR)	7.8 (3.3–25.1)	10.5 (4.9–17.9)	25.5 (7.0–47.3)	

Data were compared using χ^2 test, Student *t* test, or Mann–Whitney *U* test for qualitative or quantitative variables, respectively.

Abbreviations: Ab, antibody; Anti-*E. histolytica*, anti *Entamoeba histolytica* antibody; HBV, hepatitis B virus; HCV, hepatitis C virus; IA, invasive amebiasis; IA, invasive amebiasis; IQR, interquartile range; MSM, men who have sex with men; SD, standard deviation; TPHA, *Treponema pallidum* hemagglutination.[a]Extraintestinal cases include one case of appendicitis and 6 cases of liver abscess.

(confirmed by identification of erythrophagocytic trophozoites in surgically removed specimen), amebic liver abscess in 6, and amebic colitis in 11 (confirmed by identification of erythrophagocytic trophozoites in stool samples). The median anti-*E. histolytica* titer at baseline was significantly higher among patients who developed invasive amebiasis than that among those who did not, but the other clinical and laboratory parameters were not different between the 2 groups (Table 2). Although no significant differences in the frequency of invasive amebiasis were evident in patients with ×100 ($P = .77$) and ×200 ($P = .18$) anti-*E. histolytica* titers at baseline, compared with negative anti-*E. histolytica* patients (<×100), the frequency was higher in patients with ×400 ($P < .001$), ×800 ($P = .025$), and ≥×1600 ($P < .001$) anti-*E. histolytica* titers at baseline, compared with negative anti-*E. histolytica* patients. Univariate and multivariate analyses also showed that future development of invasive amebiasis correlated only with high titer of anti-*E. histolytica* antibody at baseline (≥×400: Univariate, HR: 20.985, 95% confidence interval [CI], 8.085–54.467; multivariate, HR: 22.079, 95% CI, 7.964–61.215) (Table 3). Furthermore, the risk of development of invasive amebiasis was significantly higher in the high anti-*E. histolytica* titer group (patients with anti-*E. histolytica* titer ≥×400 at baseline) than in the low anti-*E. histolytica* titer group (patients with anti-*E. histolytica* titer ≤×200 at baseline; log-rank test: $\chi^2 = 80.203$, $P < .001$, Kaplan-Meier estimate, Figure 2). Moreover, most patients of the high anti-*E. histolytica*

Table 3. Risk Analysis for Development of Invasive Amebiasis by Cox Proportional Hazard Regression Model

	Univariate Analysis		Multivariate Analysis	
	HR (95% CI)	*P* Value	HR (95% CI)	*P* Value
older age (by 1 y)	0.989 (.947–1.033)	.624		
Japanese nationality	1.334 (.305–5.840)	.702		
Male sex	21.884 (.002–241297.39)	.516		
MSM	4.318 (.573–32.518)	.156	4.048 (.488–33.584)	.195
TPHA test-positive	0.901 (.348–2.335)	.831		
HBV exposure-positive	2.183 (.778–6.124)	.138	1.839 (.644–5.249)	.255
HCV Ab-positive	0.047 (.000–2697.344)	.584		
Anti-*E. histolytica* titer ≥×400	20.985 (8.085–54.467)	<.001	22.079 (7.964–61.215)	<.001

The Cox proportional-hazards regression analysis was used to estimate the impact of anti-*E. histolytica* titer at baseline on the incidence of invasive amebiasis. The impact of basic clinical characteristics, such as sexuality and serology status of other STIs, was estimated with univariate Cox proportional hazards regression. Multivariate Cox hazards regression analysis using variables identified in univariate analysis with *P* values of < .20. In all analyses, statistical significance was defined as *P* value of < .05.

Abbreviations: Ab, antibody; Anti-*E. histolytica*, anti *Entamoeba histolytica* antibody; CI, confidence interval; HBV, hepatitis B virus; HCV, hepatitis C virus; IA, invasive amebiasis; IA, invasive amebiasis; IQR, interquartile range; MSM, men who have sex with men; TPHA, *Treponema pallidum* hemagglutination.

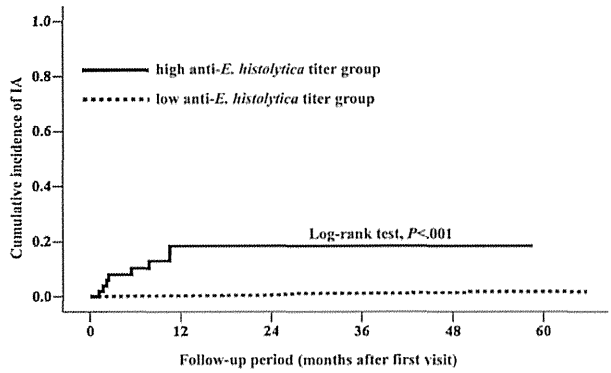

Figure 2. Incidence of invasive amebiasis in low and high anti-*E. histolytica* titer groups. Differences in the time from first visit to the diagnosis of invasive amebiasis (IA) between the low anti-*E. histolytica* titer group (≤×200 at baseline) and high anti-*E. histolytica* titer group (≥×400 at baseline) were analyzed by Kaplan-Meier method. Log-rank test was used to determine the statistical significance. Abbreviations: Anti-*E. histolytica*, anti-*Entamoeba histolytica* antibody; IA, invasive amebiasis.

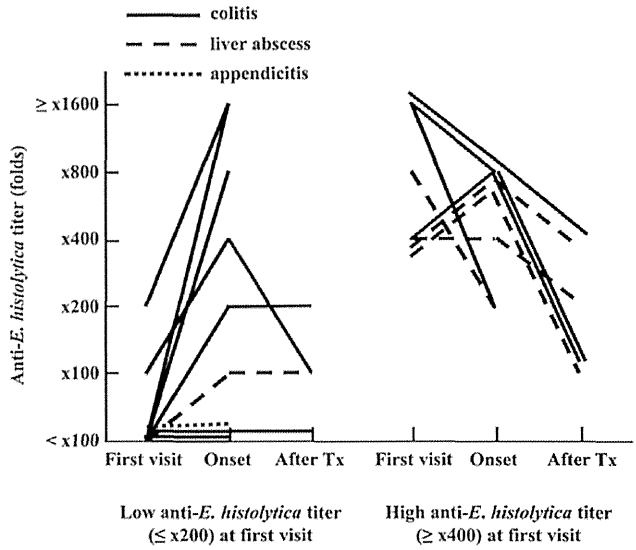

Figure 3. Anti-*E. histolytica* titer before and after diagnosis of invasive amebiasis. Anti-*E. histolytica* titer at the onset of IA was compared to that at baseline (first visit to the clinic) by Wilcoxon signed-rank test. Anti-*E. histolytica* titers after treatment were measured at 219 days [range: 174–252] and 367 days [272–841] after the completion of treatment of patients with low and high anti-*E. histolytica* titer at first visit, respectively. Abbreviations: Anti-*E. histolytica*, anti-*Entamoeba histolytica* antibody; IA, invasive amebiasis.

titer group developed invasive amebiasis during the first year of follow-up, whereas those of the low anti-E. histolytica titer group developed this complication more lately and new cases of invasive amebiasis were diagnosed throughout the follow-up period.

Transitional Changes in Anti-*E. histolytica* Titer Among Patients Who Developed Amebiasis

The median anti-*E. histolytica* titer was significantly higher at the onset of invasive amebiasis than that at first visit in patients with low baseline anti-*E. histolytica* titer (≤×200; $P = .028$, Wilcoxon signed-rank test) (Figure 3). In contrast, the median anti-*E. histolytica* titers at these 2 time points were not different in patients with high baseline anti-*E. histolytica* titer (≥×400; $P = .18$, Wilcoxon signed-rank test). Serum samples taken after nitroimidazole treatment (median time from the commencement of treatment 289 days [range 174–841]) were available in 10 patients. Anti-*E. histolytica* titers were lower after the treatment in 7 of the 10 patients, compared with the baseline values. To define the natural decay of anti-*E. histolytica*, we measured serum anti-*E. histolytica* titers at 9 months after study enrollment in 37 patients with high anti-*E. histolytica* titer at baseline but did not develop invasive amebiasis during the study period. The titers were lower, or similar to the baseline in 19 and 15 patients, respectively, whereas the remaining 3 patients showed 2-fold increase in the titer.

DISCUSSION

In the present study, the seroprevalence of anti-*E. histolytica* antibody among HIV-1-infected patients was 21.3%, which was much higher than those reported in other developed countries where amebiasis is considered as an STI [3, 9, 23, 24]. In addition, our results showed that sexually active MSM tend to be seropositive for *E. histolytica* infection, in agreement with previous studies from our group [27, 28].

The pathogenesis of amebiasis, such as incubation period after cyst ingestion and the mechanism of spontaneous remission, remains unclear. Although previous study showed anti-*E. histolytica*-positive children were more susceptible to *E. histolytica* infection than their seronegative counterparts [31], the clinical significance of anti-*E. histolytica* seropositivity and its titer in asymptomatic individuals had not been fully assessed. We measured serum anti-*E. histolytica* immunoglobulin M (IgM) levels in 18 patients at the onset of invasive amebiasis [32], but the level was detectable only in 3 patients with amebic colitis and 1 patient with liver abscess. The present study demonstrated that patients with high anti-*E. histolytica* titer (≥×400) at first visit developed invasive amebiasis much more frequently than those with low anti-*E. histolytica* titer (≤×200). The cumulative risk for invasive amebiasis among patients with high anti-*E. histolytica* titer at baseline rapidly increased during the first one year of follow-up but plateaued thereafter, suggesting that exacerbation of subclinical amebiasis occurs frequently within one year in these patients. On the other hand, the cumulative risk for invasive amebiasis among patients with low anti-*E. histolytica* titer at baseline increased more slowly and

developed at the same pace throughout the follow-up period, suggesting that the invasive amebiasis in these patients represented new infection rather than exacerbation of subclinical infection. The median anti-*E. histolytica* titer at the onset of invasive amebiasis in patients of high anti-*E. histolytica* titer group was not higher than that at first visit, whereas the titer increased at the onset compared with that at baseline in low anti-*E. histolytica* titer group. In addition, uni- and multivariate analyses identified high titer of anti-*E. histolytica* antibody at baseline as the only significant risk factor for future development of invasive amebiasis; seropositivity to other STIs was not a significant factor. These results add support to the aforementioned hypothesis regarding the difference in the pathology of invasive amebiasis between the high and low anti-*E. histolytica* groups. In this study, 15 asymptomatic but anti-*E. histolytica*-positive patients were treated with metronidazole at first visit (excluded from the follow-up analysis study), and none of them developed invasive amebiasis (median follow-up period, 11.7 months), suggesting the potential effectiveness of preemptive therapy for asymptomatic individuals with high anti-*E. histolytica* titer.

In conclusion, our results showed a relatively high prevalence of amebiasis in HIV-1-infected individuals in Japan, and that subclinical amebiasis is common among these individuals. The results emphasize the difficulty of disease control in not only individual patients with amebiasis but also in epidemiological control of this condition due to the long duration of subclinical infection of *E. histolytica*. Anti-*E. histolytica* testing for high-risk individuals could be helpful in early diagnosis of subclinical amebiasis, and early treatment of patients with such infection could prevent the development of invasive amebiasis and the transmission to others in the same community. Further studies to clarify the pathogenesis of invasive amebiasis are warranted.

Notes

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HIV protease inhibitor Lopinavir induces apoptosis of primary effusion lymphoma cells via suppression of NF-κB pathway

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ABSTRACT

Primary effusion lymphoma (PEL) is a non-Hodgkin lymphoma that occurs predominantly in patients with advanced AIDS. In this study, we examined the effect of HIV protease inhibitors, Lopinavir (LPV), Ritonavir (RTV) and Darunavir (DRV) on PEL cell lines *in vitro* and *in vivo*. LPV and RTV, but not DRV induced caspase-dependent apoptosis and suppressed NF-κB activity by inhibiting IKK phosphorylation in PEL cells. In a PEL xenograft mouse model, LPV significantly inhibited the growth and invasion of PEL cells. These results suggest that LPV may have promise for the treatment and prevention of PEL, which occurs in HIV/AIDS patients.

1. Introduction

Primary effusion lymphoma (PEL) is a subtype of non-Hodgkin B cell lymphoma that mainly presents in patients with advanced AIDS, but is sometimes also found in immunosuppressed patients, such as those who have undergo organ transplantation [1,2]. Among AIDS-related NHLs, PEL generally has an extremely aggressive clinical course with a median survival of only 6 months [2,3]. PEL usually presents as a lymphomatous effusion in body cavities and is caused by Kaposi sarcoma-associated herpes virus (KSVH/HHV-8) [1]. A number of constitutively activated signaling pathways play critical roles in the survival and growth of PEL cells. These include nuclear factor (NF)-κB, JAK/STAT and PI3 kinase [4–6]. KSHV/HHV-8 encodes a virus Fas-associated death domain-like interleukin-1β-converting enzyme (FLICE) inhibitory protein (vFLIP) that has the ability to activate the NF-κB pathway [7–9]. vFLIP has been shown to bind to the IKK complex to induce constitutive kinase activation [10] and, as a result, PEL cells have high levels of nuclear NF-κB activity, whereas inhibition of NF-κB induces apoptosis in PEL cells [5,11]. These studies support the idea that vFLIP-mediated NF-κB activation is necessary for the survival of PEL cells and that this pathway represents a target for molecular therapy for this disease.

HIV-1 protease inhibitors (HIV-PIs) have been successfully used in the treatment of HIV-1 infection. Incorporation of HIV-PIs in combination antiretroviral therapy (cART) has significantly reduced morbidity and mortality and prolonged the lifespan of patients with HIV infection. However, HIV-PIs have been shown to directly affect cell metabolism, interfere with host proteases and induce metabolic abnormalities such as insulin resistance, lipodystrophy, and hyperlipidemia, even though they were designed to selectively interfere with the catalytic site of HIV protease. Recently, HIV-PIs have become a focus of attention for having anti-tumor effects [12]. HIV-PIs have been shown to block angiogenesis, tumor cell invasion and tumor cell growth, and to induce endothelial reticulum stress, autophagy and tumor cell apoptosis both *in vivo* and *in vitro* [13–15]. Interestingly, the mechanisms of these anti-tumor effects are different with each HIV-PI, indicating that, although classified together, HIV-PIs are quite distinct compounds [16].

Ritonavir (RTV) has been shown to inhibit the chymotrypsin-like activity of the 20S proteasome and to activate the chymotrypsin-like activity of the 26S proteasome conversely [17–19]. RTV also has been reported to inhibit the transactivation of NF-κB induced by activators such as TNFα, HIV-1 Tat protein and the human herpesvirus 8 protein ORF74 [20]. It is possible that inhibition of NF-κB activation by RTV is linked to additional pathways other than proteasome inhibition. HIV-PIs also have been shown to have direct antiangiogenic and antitumor activity [12]. Recently, it was reported that RTV inhibits the growth and infiltration of ATL cells through targeting NF-κB [14,21]. Lopinavir (LPV) is a frequently used HIV-PI, but only a few antitumor effects have been reported [22]. Recently, a second generation HIV-PI,

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Darunavir (DRV), was developed. DRV has high activity against HIV protease and, as a result, DRV does not lead to tolerance induction [23–25], and since DRV is designed to specifically bind to HIV protease, the adverse effects are less frequent compared with other HIV-PIs.

In the present study, we investigated the antitumor activity of HIV-PIs against human PEL cell lines *in vitro* and *in vivo*. We found that RTV and LPV inhibit constitutively active NF-κB, leading to PEL apoptosis. In contrast, a second generation HIV-PI, DRV, has little effect against PEL cells. Our findings provide the experimental basis for utilizing HIV-PIs against tumors in HIV-1-infected individuals.

2. Materials and methods

2.1. Cell lines and reagents

The human PEL cell lines, BCBL-1 (obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH) [26], BC-1 [27], BC-3 [28], (obtained through ATCC International Essentials of Life Science Research, USA) and TY-1 [29] were maintained in RPMI1640 supplemented with 10% heat-inactivated fetal calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) in a humidified incubator at 37 °C and 5% CO_2. Ritonavir (RTV), Lopinavir (LPV) (Abbott Labs, North Chicago, IL), Darnavir (DRV) (Pfizer, NY) and LY294002 (Cell Signaling Technology, Danvers, MA) were dissolved in DMSO. DHMEQ (a kind gift from Dr. K. Umezawa, Keio University, Japan) is a NF-κB inhibitor that acts at the level of the nuclear translocation of NF-κB [30].

2.2. Tetrazolium dye methylthiotetrazole (MTT) assay

The antiproliferative effects of HIV-PIs against PEL cell lines were measured by the methylthiotetrazole (MTT) method (Sigma, St. Louis, MO). Briefly, 2×10^4 cells were incubated in triplicate in a 96-well microculture plate in the presence of different concentrations of HIV-PIs in a final volume of 0.1 ml for 24 h at 37 °C. Subsequently, MTT (0.5 mg/ml final concentration) was added to each well. After 3 h of additional incubation, 100 μl of a solution of 0.04 N HCl were added to dissolve the crystal. The absorption values at 570 nm were determined with an automatic enzyme-linked immunosorbent assay (ELISA) plate reader (Multiskan, Thermo ElectronVantaa, Finland). Values are normalized to the untreated (control) samples.

2.3. Cell cycle analysis

For cell cycle analysis, after PEL cells were treated with HIV-PIs (25 μM) for various hours, the cells were incubated in 70% ethanol at 4 °C overnight, treated with RNase A and stained with propidium iodide (PI, 50 μg/ml). The DNA content in each cell was analyzed on LSR II flow cytometer (BD Bioscience, San Jose, CA). Data were analyzed on FlowJo software (Tree Star, San Carlos, CA).

2.4. Annexin V assay

Apoptosis was quantified using the Annexin V: FITC apoptosis MEL MEBCYTO apoptosis kit (MBL, Nagoya, Japan) [31]. Briefly, after treatment with various concentration of HIV-PIs for 12 h, cells were harvested, washed with Annexin binding buffer and then incubated with Annexin V-FITC for 15 min in the dark, and PI added before being analyzed on a LSR II cytometer.

2.5. Caspase activity measurements with flow cytometry

Active caspase 3, 8 and 9 activities were measured using APOPCYTO (MBL) according to the manufacturer's instructions. Briefly, various concentrations of HIV-PI- treated or -untreated cells (200 μl) were incubated with 2 μL substrate, FITC-DEVD-FMK was added to each well, and incubated for 60 min at 37 °C in CO_2 incubator. After incubation, cells were washed with washing buffer, 2 μg/ml PI added and analyzed by LSR II. Data were analyzed on FlowJo software for the expression of active caspase 3-, 8- and 9-positive cellular events among PI-negative (living) cells.

2.6. Western blot analysis

BCBL-1 cells with or without treatment of 40 μM LPV for 1, 3 and 6 h were collected and washed in cold PBS before the addition of 300 μl cold buffer A (10 mM HEPES KOH pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.1% NP-40, 0.5 mM DTT, 0.5 mM PMSF, 2 μg/ml pepstatin A, 2 μg/ml aprotinin and 2 μg/ml leupeptin). After incubation on ice for 10 min, the samples were vortexed for 10 s and centrifuged at 5000 rpm for 1 min and supernatant collected as a cytoplasmic sample. Nuclei were pelleted by centrifugation and washed once with buffer A. Then, 100 μl buffer C (50 mM HEPES-KOH pH 7.9, 10% glycerol, 420 mM NaCl, 5 mM MgCl2, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 2 μg/ml pepstatin A, 2 μg/ml aprotinin and 2 μg/ml leupeptin) were added to the nuclei, sonicated for 10 s 10 times and incubated on ice for 3 h. Nuclear extracts were obtained by centrifugation at 15,000 rpm for 15 min. Then the cytoplasmic protein and nuclear extracts (40 μg protein) were separated by 10% SDS–PAGE and blotted onto a PVDF membrane (GE Healthcare, Buckinghamshire, UK). Detection was performed using the ECL Plus Western Blotting Detection System (ECL; GE Healthcare). Primary antibodies used were as follows: anti-p65 (F-6), anti-I-κBα (C-21), anti-IKKαβ (H-479), anti-phospho-IKKαβ (Thr23), anti-actin (C-2), anti-γ tubulin (C-20) (Santa Cruz Biotechnology, Santa Cruz, CA), and and anti-Akt (4691), anti-phospho (Thr308)-Akt (2965), anti phospho (Ser473)-Akt (4060), anti-phospho (Ser241)-PDK1 (3438), anti-PI3 kinase (4257), anti-phospho p85 (Tyr458)/ p55 (Tyr199)-PI3 kinase (4228), anti-phospho-I-κBα (Ser32/36) (Cell Signaling Technology, Danvers, MA). Western blots were quantified using the ImageQuant LAS 4000 system (GE Healthcare). Relative density was evaluated and normalized with actin or γ tubulin.

2.7. Transient transfection and NF-κB p65 reporter gene assay

BCBL-1 was transfected with the NF-κB-LUC plasmid (Stratagene, La Jolla, CA) using the Neon transfection system (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions and incubated at 37 °C for 24 h. Cells were treated with RTV, LPV, DRV and DHMEQ for 1, 3 and 6 h, and protein was extracted and subjected to determination of luciferase activity by the Dual Luciferase Assay system (TOYO INK Corporation, Tokyo, Japan) according to the manufacturer's instructions. Firefly luciferase activity was standardized using luciferase activity by Renilla luciferase activity.

2.8. RT-PCR

Total RNA was extracted from the cells using Trizol (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was synthesized from RNA using a PrimeScript RT-PCR kit (Takara Bio, Otsu, Japan) with random primers. The PCR products were analyzed by 1.5% agarose gel electrophoresis and ethidiumbromide staining. Primer sequences were as follows: ORFK13 (v-FLIP): 5′-ATTGACATTAGGGCATCC-3′ and 5′-AAAGGAGGA GGGCAGGTT, ORF72 (v-cyclin): 5′-GATAATAGAGGCGGGCAATG-3′ and 5′-TAA AGCAGGTGTCCAAAGAA-3′, ORF73 (LANA): 5′-GAAGTGGATTACC CTGTTGT TAGC-3′ and 5′-TTGGATCTCGTCTTCCATCC-3′, ORF50 (RTA): 5′-GCC CTCTGC CTTTTGGTT- 3′ and 5′-GATGATGCTGACGGGTGTG-3′, GAPDH: 5′-CGGGAAG CTTGTGATCA ATGG-3′ and 5′-GGCAGTGATGGCATG GACTG-3′[32,33]

2.9. Xenograft mouse model

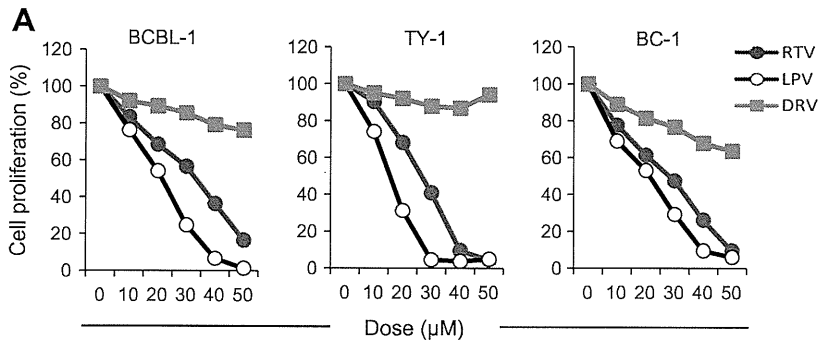
NOD/Scid/Jak3-deficient (NOJ) mice were established by backcrossing Jak3-deficient mice [34] with the NOD.Cg.-Prkdcscid strain for 10 generations [35]. NOJ male mice of 8 to 10 weeks old were housed and monitored in our animal research facility according to the institutional guidelines. All experimental procedures and protocols were approved by the Institutional Animal Care and Use Committee at Kumamoto University. NOJ mice were intraperitoneally inoculated with 1×10^7 BCBL-1 cells suspended in 100 μl PBS. The mice were then treated with intraperitoneal injections of PBS or HIV-PIs (40 μmol/kg per day). Tumor burdens were evaluated by measuring the volume of ascites.

2.10. Immunohistochemistry

To investigate the expression of KSHV/HHV-8 ORF73 (LANA) protein, tissue samples were fixed with 10% neutral-buffered formalin, embedded in paraffin and cut into 4 μm sections. The sections were deparaffinized by sequential immersion in xylene and ethanol and rehydrated in distilled water. They were then irradiated for 15 min in a microwave oven for antigen retrieval. Endogenous peroxidase activity was blocked by immersing the sections in methanol/0.6% H_2O_2 for 30 min at room temperature. Affinity-purified PA1-73N antibody [20], diluted 1:3,000 in PBS/5% bovine serum albumin (BSA), was then applied, and the sections were incubated overnight at 4 °C. After washing in PBS twice, the second and third reactions and the amplification procedure were performed using kits according to the manufacturer's instructions (catalyzed signal amplification system kit; DAKO, Copenhagen, Denmark). The signal was visualized using 0.2 mg/ml diaminobenzidine and 0.015% H_2O_2 in 0.05 mol/l Tris–HCl, pH 7.6.

2.11. Statistical analysis

All assays were performed at least in triplicate and expressed as mean values ± SD. The statistical significance of the differences observed between experimental groups was determined using Student's *t* test. $P < 0.05$ was considered significant.



B

CC50	BCBL-1	TY-1	BC-1	BC-3
RTV	35μM	27μM	28μM	32μM
LPV	22μM	16μM	21μM	20μM
DRV	>50μM	>50μM	>50μM	>50μM

Fig. 1. HIV protease inhibitors inhibit the proliferation of PEL cells. (A) PEL cell lines (BCBL-1, TY-1 BC-1 and BC-3) were incubated with 10, 20, 30, 40, 50 μM RTV, LPV DRV for 24 h. A cell proliferation assay was carried out using MTT as described in Section 2. A representative result from 3 independent experiments is shown. (B) Show the IC50 value of each HIV protease inhibitors.

3. Results

3.1. RTV and LPV cause dose-dependent inhibition of the proliferation and apoptosis of PEL cell lines

We initially sought to determine whether HIV-PI treatment leads to the inhibition of PEL cell proliferation. Four PEL cell lines (BCBL-1, TY-1, BC-1 and BC-3) were cultured in the presence of 10, 20, 30, 40, and 50 μM HIV-PIs for 24 h, and proliferation was analyzed by MTT assays. Fig. 1A shows that as the dose of RTV and LPV increased from 10 to 50 μM, cell growth inhibition increased in a dose-dependent fashion in all PEL cell lines (Fig. 1A). On the other hand, marked anti-proliferative effects were not observed by DRV at these concentrations. Fig. 1B shows the IC50 value of each HIV-PI individual. In subsequent experiments, we determined whether the observed suppressive effects of RTV and LPV in the MTT assay were due to the induction of cell cycle arrest or apoptosis. As shown in Fig. 2, 25 μM RTV and LPV treatment for 12 h induced cell cycle arrest. Next we used Annexin V and propidium iodide dual staining to detect apoptosis. Annexin-positive propidium iodide-negative fraction represents the early phase of apoptosis whereas Annexin-positive propidium iodide-positive fraction represents the late phase of apoptosis and necrosis [31]. As shown in Fig. 3A, 40 μM RTV and LPV treatment for 12 h caused apoptosis in all cell lines tested, but DRV did not induce apoptosis in these cell lines. Next, we measured the activation of caspase 3, 8 and 9 to further confirm that RTV and LPV induced apoptosis in PEL cells. As shown in Fig. 3B, RTV and LPV treatment of PEL cells induced the activation of caspase 3, 8 and 9, a hallmark of cells undergoing apoptosis.

3.2. LPV efficiently blocks the constitutive NF-κB activity of PEL cell lines

As several reports have suggested that NF-κB can act as a survival factor and is required for the proliferation of PEL cells, and PEL cells are known to induce apoptosis with inhibition of the NF-κB pathway, [5,11,36] we examined whether LPV inhibits NF-κB activation. When PEL cell lines were treated with 40 μM LPV for 1, 3 and 6hr, the amount of phosphorylated I-κBα protein was severely reduced; however, the amount of I-κBα protein was almost the same, indicating that LPV suppresses NF-κB activity by suppressing the activation of I-κBα phosphorylation (Fig. 4A). Suppression of I-κB phosphorylation blocked the nuclear translocation of NF-κB p65 and led to the accumulation of NF-κB p65 protein (Fig. 4A). Next, we fractioned nuclear protein and analyzed the expression of p65 by Western blotting (Fig. 4B) to confirm NF-κB p65 suppression by LPV. When PEL cell lines were treated with 40 μM LPV for 6 h, the amount of nuclear NF-κB p65 protein was reduced as expected, indicating that LPV suppresses NF-κB activity. To confirm that LPV could inhibit NF-κB transcriptional activity in PEL cell lines, we performed the NF-κB promoter assay. Treatment with RTV and LPV suppressed the transcriptional activity of NF-κB in all cell lines tested (Fig. 4C); however, DRV did not inhibit NF-κB activity. These results revealed that LPV blocks the constitutive NF-κB activity of PEL cells.

3.3. Inhibition of NF-κB activity is not depend on Akt pathway

Akt pathway regulates NF-κB activity and it was reported that LPV inhibits Akt pathway[37]. Therefore, we examined whether LPV suppresses IKK phosphorylation through inhibition of Akt pathway. When PEL cell lines were treated with 40 μM LPV for 1, 3 and 6 h, the amount of phosphorylated Akt protein was severely reduced. However amount of phosphorylated PI3 kinase which is upstream of Akt pathway was almost same indicated that LPV suppressed Akt phosphorylation (Fig. 5A) Furthermore, we examined whether inhibition of Akt pathway suppresses IKK phosphorylation in PEL cells. LY294002 (PI3 kinase inhibitor) treatment suppressed Akt phosphorylation but could not suppressed IKK phosphorylation (Fig. 5B), indicated that inhibition of Akt pathway cannot suppress IKK phosphorylation in PEL cells. These results indicate that suppression of IKK phosphorylation by LPV is not due to the inhibition of Akt pathway.

3.4. LPV dose not induce HHV-8 reactivation

It is well known that v-FLIP has the ability to activate the NF-κB pathway through binding to the IKK complex to induce

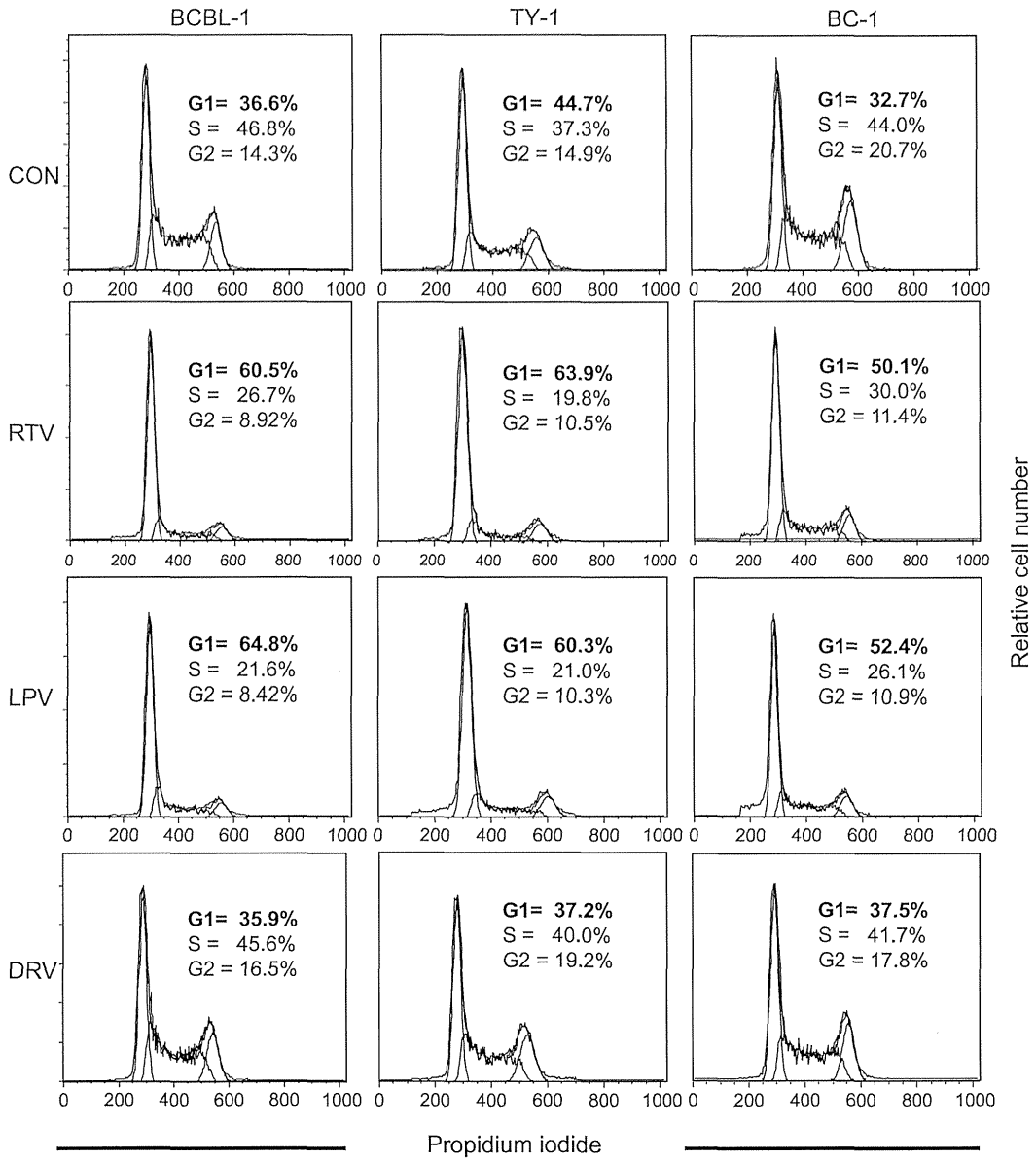


Fig. 2. HIV protease inhibitors cause cell cycle arrest of PEL cells. PEL cell lines BCBL-1, TY-1 and BC-1 were treated with RTV, LPV, DRV (25 μM) for 12 h, and DNA histograms were determined and the cell cycle was analyzed using FlowJo software. A representative result from 3 independent experiments is shown.

constitutive kinase activation [7,36,38]. Therefore, RT-PCR was performed to examine the effects of LPV on viral gene expression including v-FLIP. LPV treatment did not change both of lytic (RTA) and latent gene (v-FLIP, LANA and v-cyclin) expression (Fig. 5C), indicated that LPV have no direct effect on viral gene expression including v-FLIP.

3.5. Antitumor effect of LPV in PEL-inoculated mice

As the above results suggested the efficacy of RTV and LPV for the treatment of PEL patients, we next examined the *in vivo* effects of LPV in a PEL-inoculated mouse model. Severely immunodeficient, NOD/Scid/Jak3-deficient mice (NOJ mice) [35] were inoculated intraperitoneally with 1×10^7 BCBL-1 cells. BCBL-1 produced massive ascites within 4 weeks of inoculation (Fig. 6A). As PEL is characterized by lymphomatous effusion of serous cavities and rarely presents with a definable tumor mass [1,2], these mice are a clinically relevant PEL model. A dose of 40 μmol/kg/day LPV and DRV or PBS was administrated via intraperitoneal injection on day 3 after cell inoculation and every day thereafter for 28 days. LPV-treated mice appeared to be healthy and had a significantly lower volume of ascites than DRV or PBS-treated mouse ascites (0.47 ± 0.35 ml vs. 5.08 ± 1.52 ml, $n = 10$ each, $p < 0.001$) (Fig. 6B). Organ infiltration by tumor cells was analyzed and evaluated by hematoxylin–eosin staining and LANA immunostaining (Fig. 6C). We found that mice inoculated intraperitoneally with BCBL-1 exhibited infiltration into the lung, liver, and spleen without macroscopic lymphoma formation. The number of LANA-positive cells in LPV-treated mice was significantly reduced (0–1 cells per field magnification, 340) compared to non-treated, DRV mouse (10–20) cells per field magnification, 340) (Fig. 6D). These data indicate that LPV significantly inhibits the growth and infiltration of PEL cells *in vivo*.

4. Discussion

In the present study, we investigated the effects of three different HIV PIs, RTV, LPV and DRV, on PEL cells both *in vitro* and *in vivo*.

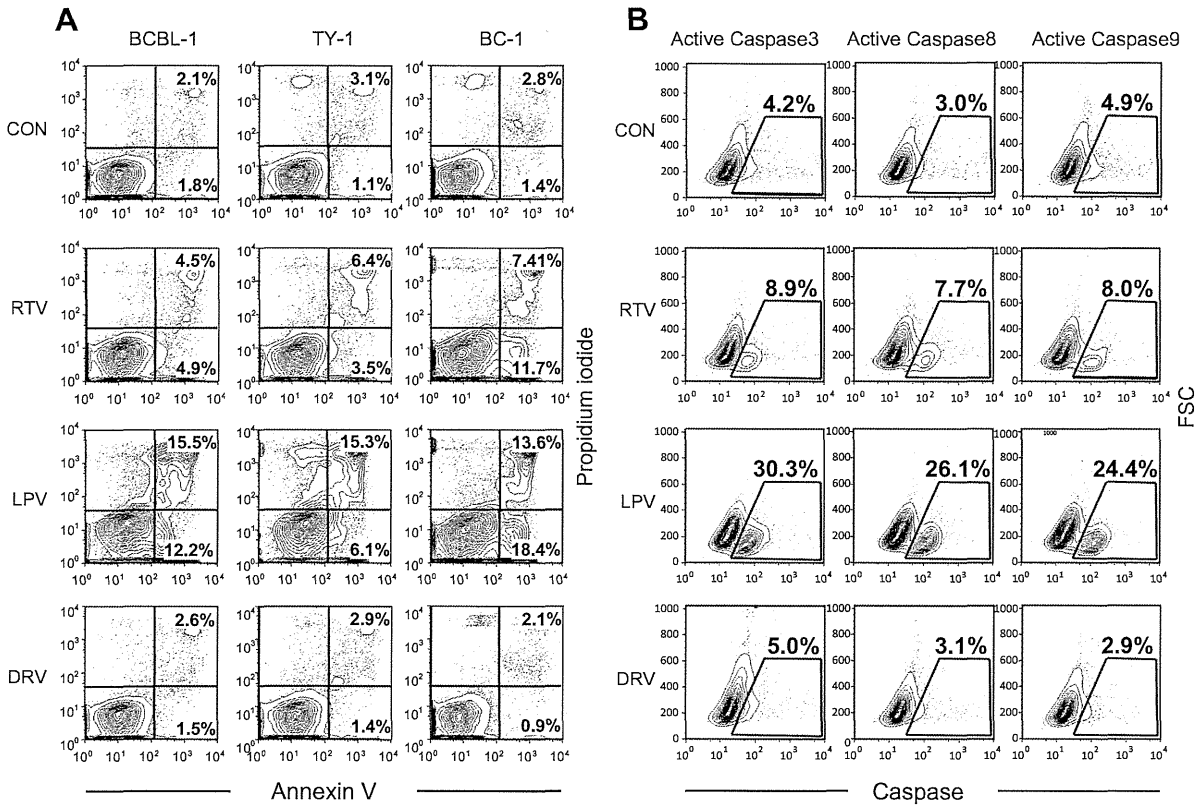


Fig. 3. HIV protease inhibitors cause apoptosis of PEL cells. (A) HIV protease inhibitors induced apoptosis as detected by Annexin V and propidium iodide dual staining. PEL cell lines BCBL-1, TY-1 and BC-1 were treated with 40 μM of RTV, LPV and DRV for 12 h and were subsequently stained with Annexin-FITC and propidium iodide before being analyzed by flow cytometry. (B) HIV protease inhibitor induces apoptosis of PEL cells via caspase-3-, 8- and 9-dependent pathway. The PEL cell line BCBL-1 was treated with RTV LPV DRV (40 μM) for 12 h and was subsequently stained with caspase-3, 8, 9 before being analyzed by flow cytometry. A representative result from 3 independent experiments is shown.

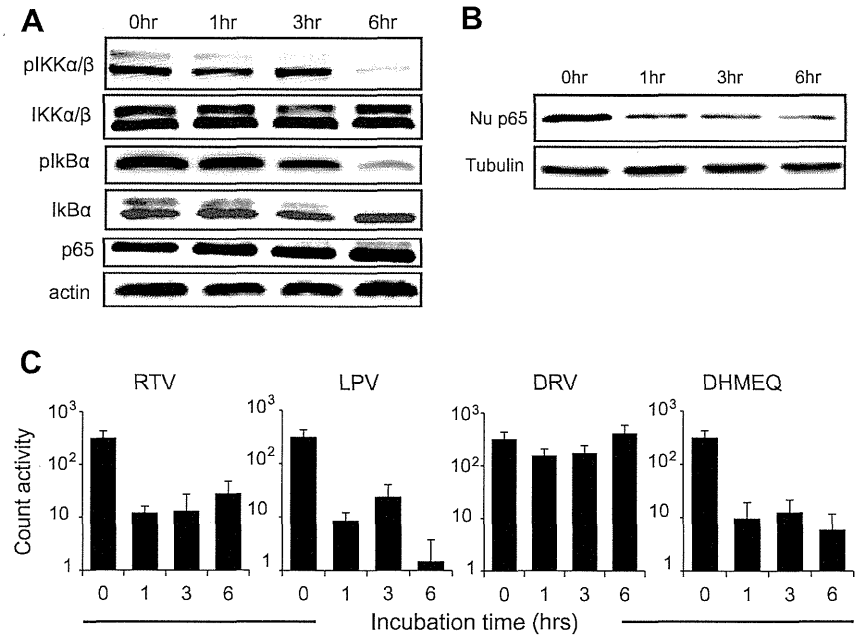


Fig. 4. Inhibitory effects of HIV protease inhibitors on the expression of NF-κB pathways. (A) The PEL cell line BCBL-1 was treated with LPV (40 μM) for 1, 3 and 6hr and cytoplasmic proteins were extracted and Western blot was performed. The numbers indicate the relative expression of each protein level normalized with actin. (B) The PEL cell line BCBL-1 was treated with LPV (40 μM) for 1, 3 and 6 h and nuclear proteins were extracted and Western blot was performed to detect NF-κB p65. The numbers indicate the relative expression of p65 normalized with γ tubulin. A representative result from 3 independent experiments is shown. (C) BCBL-1 was transfected with NF-κB-LUC plasmid and 24 h later cells were treated with 40 μM of RTV, LPV, DRV and DHMEQ for 1, 3, 6 h and protein was extracted, and firefly and Renilla luciferase were measured on a luminometer. Firefly luciferase activity was normalized to Renilla luciferase expression.

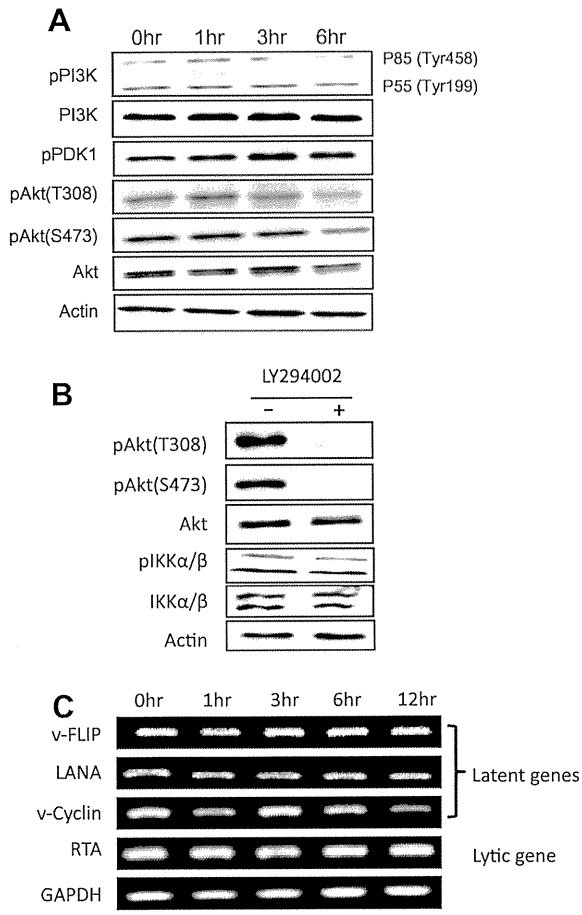


Fig. 5. Effects of LPV against upstream of IKKα and IKKβ. (A) The PEL cell line BCBL-1 was treated with LPV (40 μM) for 1, 3 and 6hr and cytoplasmic proteins were extracted and Western blot was performed. (B) The PEL cell line BCBL-1 was treated with LY294002 (50 μM) for 3hr and cytoplasmic proteins were extracted and Western blot was performed. (C) Viral gene expression after treatment with LPV (40 μM) for 1, 3 and 6hr was examined by RT-PCR.

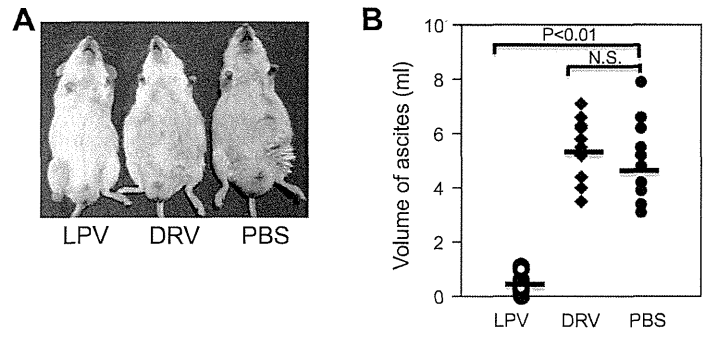
Our results showed that RTV and LPV but not DRV exhibited potent pro-apoptotic effects on PEL cells and provided evidence that such apoptosis occurs via then inhibition of NF-κB activity. These direct anti-tumor effects were shown in animal models free of viruses and immunocompetent cells such as T cells and NK cells, and in which direct anti-tumor effects of HIV-PIs that were independent of drug-mediated HIV suppression and immune reconstitution could be detected.

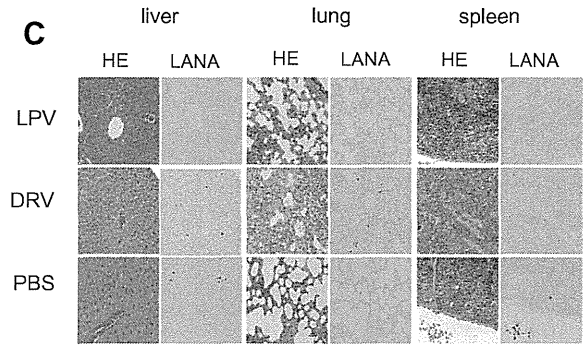
PEL is an incurable, aggressive B-cell malignancy and most patients that suffer from it respond poorly to traditional chemotherapy and develop chemoresistance. A novel strategy for treatment is needed for this aggressive lymphoma. Several agents have been tested in the search for a more effective treatment for PEL. It is now postulated that the mechanisms of lymphomagenesis involve the deregulation of several signaling pathways that may act either independently or crosstalk with each other. These include NF-κB, JAK/STAT and PI3 kinase pathways [4–6] in the case of PEL. PEL is associated with KSHV/HHV-8 infection and KSHV/HHV-8 contains a homologue of the cellular FLIP protein vFLIP, which has the ability to activate the NF-κB pathway through binding to the IKK complex to induce constitutive kinase activation. [7,36,38] Moreover, inhibition of NF-κB activity leads to the apoptosis of KSHV-infected PEL cells [5,11]. These results suggest that the NF-κB pathway is an effective target for the treatment of PEL. Activation of NF-κB is involved in various kinds of cancer development and progression [39–41] as well as in virus-associated lymphomas, indicating that NF-κB is a good molecular target for cancer treatment. HIV-PIs, such as RTV, LPV and DRV, have been successfully used in clinical treatments of HIV infection, with patients exhibiting a marked decrease in HIV viral load and a subsequent increase in CD4+ T-Cell counts [42–45]. Antitumor effects of HIV-PIs are expected, since patients treated with HAART but failing in CD4 T cell recovery still show a significantly lower risk of AIDS-related malignancies [46,47]. In fact, antitumor effects and the induction of apoptosis by HIV-PIs have been reported in the last 10 years [14,19,21]. In our study, we demonstrated that RTV and LPV are able to suppress the growth of PEL cells and induce apoptosis via the inhibition of NF-κB activity, especially by blocking the phosphorylation of I-κBα (Fig. 4A).

It was previously reported that HIV-PIs inhibit proteasome activity [21,48], and proteasome inhibition leads to the accumulation of I-κBα resulting in inhibition of NF-κB pathway. However, we could not detect any I-κBα protein accumulation in our study (Fig. 4A), indicating that LPV induced NF-κB inhibition is not due to the proteasome inhibition. In the present study, we showed that LPV inhibited IKKα and IKKβ phosphorylation (Fig. 4A). IKKα and IKKβ have been pursued by many groups as targets for the development of therapeutic agents to be used for the treatment of cancer, as well as inflammatory and metabolic diseases [49]. Akt and TAK1 have been identified as upstream kinase for IKKα and IKKβ and it was already reported that several HIV-PIs including LPV inhibit Akt pathway [37]. In present study, we also detected that LPV suppressed Akt pathway (Fig. 5A). However, specific inhibitor of Akt phosphorylation (LY294002) could not suppress IKKα and IKKβ phosphorylation in PEL line (Fig. 5B). It was also reported that TAK1 specific inhibitor cannot inhibit v-FLIP induced IKK kinase activation [50]. Taken together, Akt and TAK1 are not important molecule for v-FLIP mediated IKK phosphorylation in PEL cells. Actually, v-FLIP has the ability to activate NF-κB pathway thorough the direct binding to IKKγ [7,36,38]. If LPV induced HHV-8 lytic activation, v-FLIP expression is reduced and suppress NF-κB pathway. However, lytic activation of HHV-8 was not observed with the addition of LPV(Fig. 5C). v-FLIP is not a kinase that activates the IKK complex by inducing its phosphorylation. Instead, v-FLIP activates the complex by direct interaction via a mechanism believed to involve a conformational change of IKK complex [51]. Our findings emphasize the implication of v-FLIP for PEL survival and LPV might inhibit IKK phosphorylation by blocking of the v-FLIP and IKKγ binding at least independent of Akt.

Although designed to target only the HIV protease, HIV protease inhibitors are known to cause toxicity in patients, such as insulin resistance and lipodystrophy, suggesting that HIV protease inhibitors have other targets in mammalian cells. In fact, one of the most potent HIV-PIs, Nelfinavir, exerted pleiotropic biochemical and cellular effects on cancer cells that included the induction of endoplasmic reticulum (ER) stress, autophagy, and apoptosis *in vitro* and *in vivo* [52]. These preclinical studies suggested the idea that HIV-PIs can reduce the risk of cancer among HIV-1-infected persons. As HIV-1-infected patients have a higher risk of cancers than the uninfected population, and the complication of cancer is becoming one of the most important life-threatening events, if HIV-PIs can reduce the risk of cancer, this is a benefit for HIV-1-infected patients. Until now, only one cohort study has been performed and the risk of cancer was the same among those using HIV-PI or HIV-PI-sparing regimens [53]. Additional studies using large registries and post-marketing surveillance are needed to provide further data on the possible relationship between HIV-PIs and cancer inhibition among HIV-infected persons.

DRV was designed to form robust interactions with the protease enzyme from many strains of HIV-1, including strains from patients with multiple resistance mutations to HIV-PIs. DRV did not show these antitumor effects or inhibitory effects on the NF-κB





D

No.	drug	spleen	liver	lung
4096	LPV	-	-	+/-
4097	LPV	-	-	-
4098	DRV	++	++	++
4099	DRV	++	++	++
4100	PBS	+	+	+
4101	PBS	++	+	+

++: 1 area(x100)>10 points
+: 1 area (x100) 1-9 points
-: no points

Fig. 6. Treatment of NOD/Scid/Jak3-deficient mice with HIV protease inhibitor LPV suppresses the development and metastasis of KSHV-associated lymphoma *in vivo*. (A) A photograph of LPV, DRV-treated and non-treated ascites-bearing mice 4 weeks after being inoculated with BCBL-1 intraperitoneally. (B) Quantization of volume of ascites. (C) Hematoxylin-eosin staining and immunohistochemical staining using anti-LANA (PA1–73 N antibody) was performed to detect BCBL-1 in liver, lungs and spleen. (D) Quantification of BCBL-1 metastasis.

pathway in our study. DRV has very high activity against HIV-1 protease [23–25], but not against mammalian proteases, indicating the lack of anti-tumor effects as well as few adverse effects. In fact, clinical studies have demonstrated that DRV has more potent antiviral efficacy with more favorable lipid profiles that other HIV-PIs[54]. Thus, appropriate and creative use of HIV-PIs is required based on their actions and adverse effects.

In conclusion, we have shown the ability of HIV-PI, RTV and LPV to induce cell death through blocking the NF-κB pathway in PEL cells. Our study provides a rationale for a clinical trial of HIV-PIs in patients with PEL and other NF-κB-activated tumors. Further investigations aimed at determining the efficacy of HIV-PIs are warranted and may lead to the development of new effective therapies for this intractable lymphoma.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgements

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INTERNAL MEDICINE

CASE REPORT

Three Cases of Concurrent Infection with *Mycobacterium tuberculosis* and *Cryptococcus neoformans*

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Abstract

Impaired cellular-mediated immunity is a known risk factor for both tuberculosis and cryptococcosis. However, pulmonary cryptococcosis associated with pulmonary tuberculosis is rare. We herein describe three cases of concurrent infection with *Mycobacterium tuberculosis* and *Cryptococcus neoformans*. All patients had underlying diseases; all three had uncontrolled diabetes mellitus, and other underlying diseases were liver cirrhosis, malignancy, and rheumatoid arthritis requiring long-term steroid use. We also review other relevant reports.

Key words: pulmonary cryptococcosis, pulmonary tuberculosis, co-infection

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Introduction

Impaired cellular-mediated immunity is a known risk factor for both tuberculosis and cryptococcosis. However, only a handful of cases of pulmonary cryptococcosis associated with pulmonary tuberculosis have been reported (1-5). We herein report three cases of tuberculosis and cryptococcosis co-infection in patients treated at Nagasaki University Hospital and also carry out a review of other relevant reports.

Case Reports

Case 1

A 65-year-old woman with type II diabetes that had remained untreated for an extended duration was admitted to a local hospital with appetite loss, general fatigue of seven days' duration, and severe dyspnea of two days' duration. Chest radiography revealed a left pneumothorax, small bilateral granular shadows, and consolidation with a cavity in the left upper field. We immediately performed drainage of the thoracic cavity. Nodular shadows with cavities and diffuse small opacities were seen on thoracic computed tomography (CT) (Fig. 1). She was transferred to our hospital the next day. She did not have a past history of any evident exposure to tuberculosis.

Her physical state on examination revealed malnutrition. Her body temperature was 38.0°C, her blood pressure was 122/64 mmHg, her heart rate was 92/min, and she had mild pretibial pitting edema. Moist rales were heard in both lung fields, but no heart murmur was audible. The patient's abdominal examination was normal, and the neurological examination revealed no nuchal rigidity, cranial nerve deficit, or papilledema. Her tendon reflexes were normal without any pathological reflexes.

Laboratory studies showed severe inflammation: her white blood cell (WBC) count was 6,000/μL with a neutrophil

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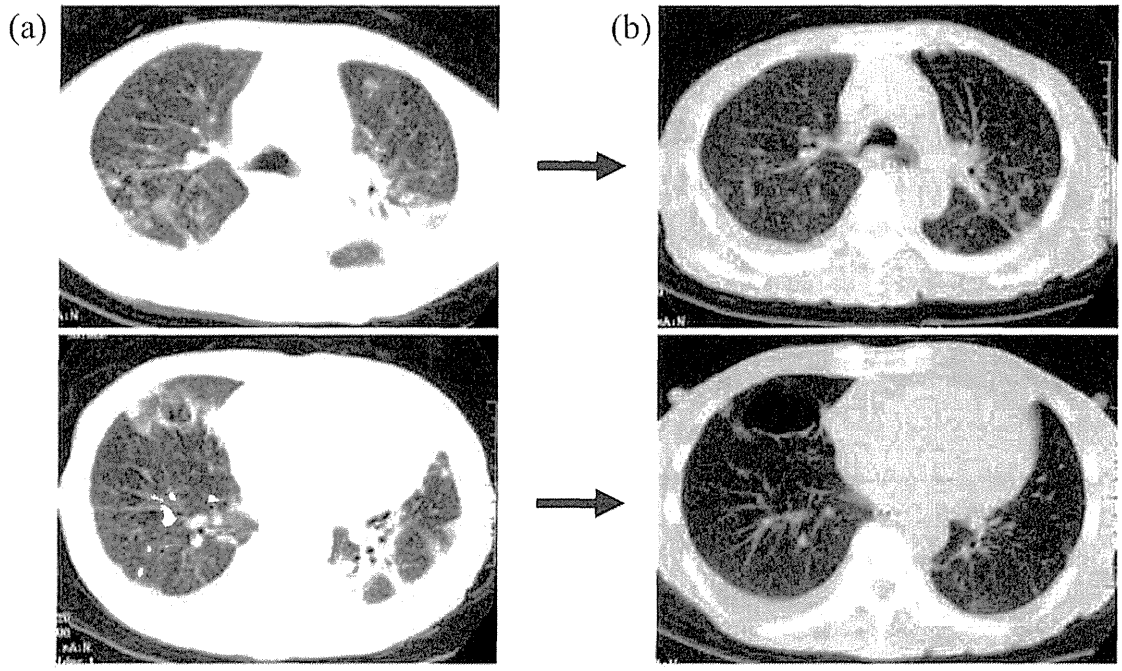

Figure 1. Thoracic CT images of case one. (a) on admission and (b) on day 120 after admission.

count of 96%, and her C-reactive protein level (CRP) was 30.35 mg/dL. Her erythrocyte sedimentation rate (ESR) was 86 mm/h. Blood chemistry data revealed a low protein level (5.2 g/dL), a low albumin level (2.5 g/dL), and a low cholinesterase level (72 IU/L; normal range 200-450 IU/L), suggesting nutritional deficiency. Fasting blood sugar (FBS) and glycated hemoglobin (HbA1c) levels were 387 mg/dL and 13.6%, respectively, suggesting poorly controlled diabetes mellitus. The QuantiFERON test (QFT) and human immunodeficiency virus (HIV) screening test were not performed. A blood gas analysis showed a PaO_2 of 63.5 mmHg and a $PaCO_2$ of 49.4 mmHg under 6 L/min O_2 through a mask.

Acid-fast staining of her sputum revealed a few beaded bacilli (Gaffky 7). Polymerase chain reaction (PCR) for *Mycobacterium tuberculosis* was positive. She was diagnosed with pulmonary tuberculosis, and antituberculosis chemotherapy was initiated, consisting of isoniazid (INH) 400 mg/day, rifampicin (RFP) 450 mg/day, pyrazinamide (PZA) 1.2 g/day, and streptomycin (SM) 0.75 g every day for two weeks, then three times a week thereafter. *M. tuberculosis* was not detected in her bone marrow, urine, or blood. Although her chest radiography findings improved and her sputum culture became *M. tuberculosis*-negative, a low-grade fever and headache persisted. Therefore, meropenem was concurrently administered under a tentative diagnosis of double-bacterial infection. One month after the start of administration, inflammatory response was improved (CRP: 1.85 mg/dL). With respect to the secondary pneumothorax, the trocar catheter was removed.

Approximately 50 days after admission, she exhibited a low-grade fever of between 37 and 37.8°C. Her serum cryptococcal antigen titer was positive at 1:32 (Serodirect® 'Eiken' Cryptococcus, Eiken Co., Tokyo, Japan). She complained of a headache, but no nuchal rigidity was observed. Lumbar puncture was performed, and cerebrospinal fluid (CSF) cryptococcal antigen was positive at a titer of 1:8. However, the total nucleated cell count was not increased, and *Cryptococcus neoformans* was not isolated.

Thereafter, a thoracic CT revealed a new shadow in the right S1a region, and a bronchoscopy was performed; however, no pathogenic bacteria were detected. On day 90 after admission, the patient's fever returned, and her level of consciousness deteriorated. A neurological examination revealed nuchal rigidity.

A second lumbar puncture was performed to rule out meningitis, and her CSF cryptococcal antigen was positive at a titer of 1:512. Examination of her CSF showed a total nucleated cell count of 250/3 per mm^3 with 80% mononuclear cells. An India ink mount of CSF revealed a few encapsulated yeast cells, which was suggestive of *C. neoformans*. Therefore, we diagnosed pulmonary tuberculosis coinfection with cryptococcal meningoencephalitis.

The patient was started on combination therapy with amphotericin B deoxycholate (AMPH-B) (0.1 mg/kg/day during the first day of therapy, 0.5 mg/kg/day from the second day, and continuation with 1.0 mg/kg/day) and fluconazole (FLCZ) (400 mg/day). Thereafter, her consciousness level transiently improved, and pyretolysis was observed. Approximately two months later, the number of cells in her CSF had normalized. As a result, the antifungal agents were therefore discontinued (Fig. 2).

However, on day 100 after admission, aspiration pneumonia, pyelonephritis, *Pseudomonas aeruginosa*-related sepsis,

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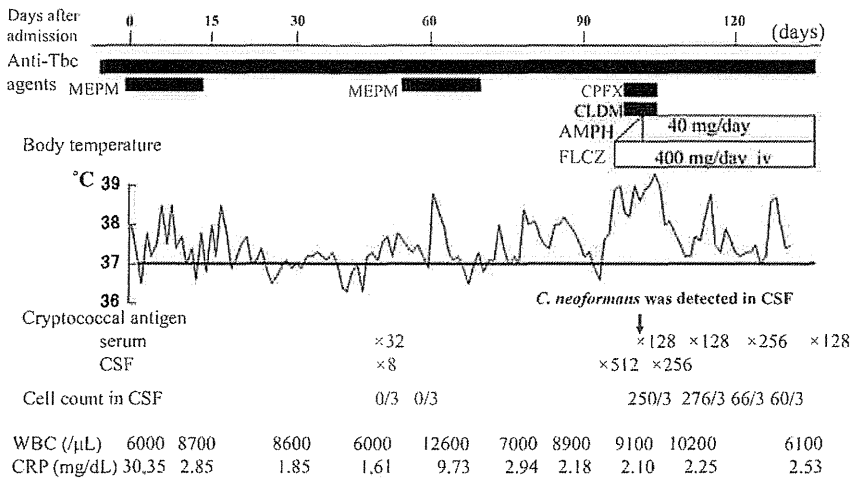

Figure 2. Clinical course of case 1. Tbc: tuberculosis, MEPM: meropenem, CPFX: ciprofloxacin, CLDM: clindamycin, AMPH: amphotericin, FLCZ: fluconazole, iv: intravenous, CSF: cerebrospinal fluid, WBC: white blood cell, CRP: C-reactive protein

pneumonia, and herpes zoster developed. On day 175 after admission, fever, deterioration of respiratory condition, and kidney/liver dysfunction appeared. On day 180 after admission, the patient died of respiratory failure due to aspiration pneumonia, although she had been provided with mechanical ventilation for a few days. An autopsy did not detect any *C. neoformans* in the lungs or other organs.

Case 2

Case 2 was a 56-year-old man with a 10-year history of hypertension, type II diabetes, and liver cirrhosis (hepatitis C). Although he had no respiratory symptoms, multiple nodular opacities were found in both upper lung fields on a routine follow-up chest radiograph. Because a serum cryptococcal antigen test was positive at a titer of 1:4, he was transferred to our hospital. He did not have a past history of tuberculosis, but his mother had died of pulmonary tuberculosis.

On admission, no abnormal breath sounds were detected and no heart murmur was audible. An abdominal examination detected hepatomegaly with an irregular surface that elastic-hard on palpation. The neurological examination results were not remarkable. No nuchal rigidity was observed. His body temperature was 35.6°C.

The patient's laboratory studies showed a WBC count of 7,900/μL with a neutrophil count of 85%, a red blood cell (RBC) count of 376×10^4/μL, and a blood platelet count of 8.6×10^4/μL. His CRP level was 0.48 mg/dL, and ESR was 65 mm/h. Blood chemistry data revealed a low protein level (6.2 g/dL), a low albumin level (2.8 g/dL), and a prothrombin time international normalized ratio (PT-INR) of 1.10. The patient was positive for hepatitis C virus antibody. His FBS and HbA1c levels were 174 mg/dL and 9.6%, respectively, suggesting poorly controlled diabetes mellitus. Protein induced by vitamin K absence (PIVKA) level was 315 mAU/mL. A blood gas analysis showed a PaO_2 of 77.3 mmHg and a $PaCO_2$ of 37.7 mmHg in room air. As a subset of lymphocytes, the ratio of CD4 to CD8 cells was 0.78 on admission. An HIV test was not conducted at that time; however, a tuberculin reaction test showed strong positivity. QFT testing was not performed.

A chest CT scan indicated the presence of multiple nodular shadows; some shadows showed cavitary lesions, and some opacities were located adjacent to the pleura (Fig. 3a).

A cryptococcal serum antigen test was positive (1:4), and *C. neoformans* was cultured from his sputum. Furthermore, acid-fast staining of his sputum revealed a few beaded bacilli (Gaffky 3). The PCR for *M. tuberculosis* was positive, but the CSF culture was negative for *C. neoformans* and mycobacteria. The cryptococcal antigen was negative in the CSF. Based on these findings, antituberculosis chemotherapy was initiated.

After starting antituberculosis chemotherapy, the patient developed an allergic reaction to INH, and a drug lymphocyte stimulation test was positive against INH. Therefore, administration of INH was started using the hyposensitization method. INH at 450 mg/day, RFP at 450 mg/day, SM at 750 mg twice per week, and levofloxacin at 600 mg/day were continuously administered. *M. tuberculosis* was undetectable from sputum approximately one month after the start of treatment. The patient's blood sugar control improved after treatment was switched to insulin injection (FBS: 80 mg/dL, HbA1c: 5.1%).

We selected antituberculosis chemotherapy to avoid an interaction between antituberculosis agents and azole antifungal agents. After antituberculosis chemotherapy, the administration of antifungal agents was scheduled. As a subset of lymphocytes, the ratio of CD4 to CD8 cells was 3.85 at four months after admission, suggesting a Th1-dominant immunoreaction (Fig. 4). A thoracic CT scan showed improvement nine months after admission (Fig. 3b). His cryptococcal serum antigen titer became negative at nine months after

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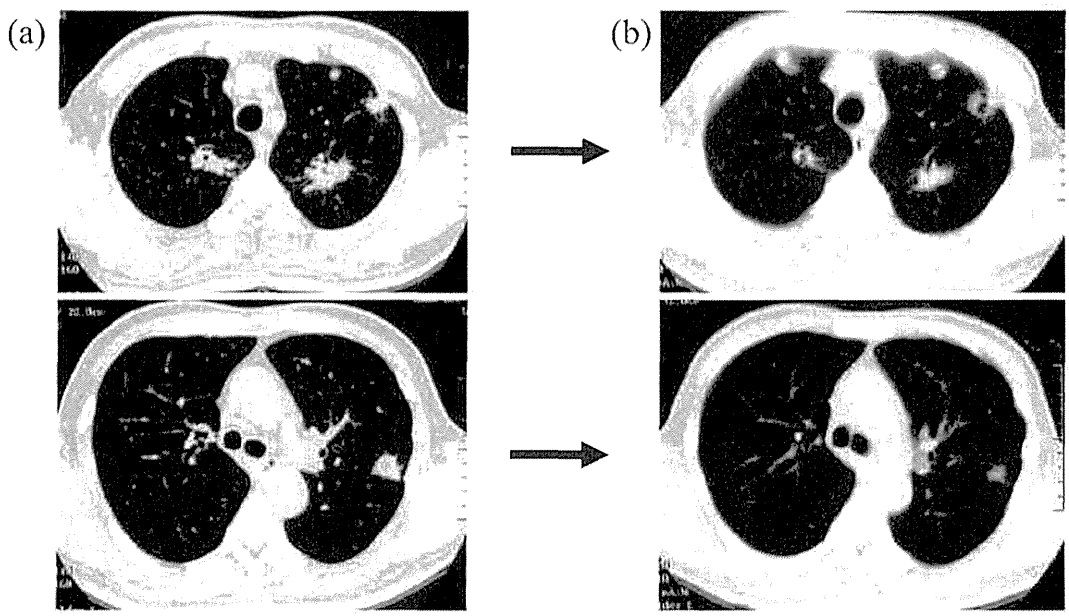

Figure 3. Thoracic CT images of case 2. (a) on admission and (b) nine months after admission.

admission.

After one year, the patient developed hepatocellular carcinoma (HCC) and visited our hospital again. The cryptococcal antigen was not detected at re-admission, and no new pulmonary shadows were present. He died of HCC one year after re-admission.

Case 3

An 83-year-old woman with rheumatoid arthritis had been treated with prednisolone (PSL, 5 mg/day) for several years in another hospital. She did not have a past history of evident exposure to tuberculosis, and prophylactic antimycobacterial agents were not administered.

After an increase of the PSL daily dose to 40 mg to address worsening of arthralgia, a chest radiograph revealed a cavitary lesion in the right upper lobe, and diffuse small granular shadows were observed in both lungs (Fig. 5). Acid-fast staining of her sputum showed bacilli (Gaffky 3), and the PCR analyses for *M. tuberculosis* were positive in samples of CSF, urine, and stool. Miliary tuberculosis was confirmed. Because of anemia due to digestive tract bleeding, her unconscious state, and other aspects of her general condition, she was transferred to our hospital.

On arrival, she was in a comatose state [Japan Coma Scale (JCS) III-300 or Glasgow Coma Scale score of three]. Coarse crackles were heard in both lung fields, and systolic heart murmurs were audible. An abdominal examination indicated moderate ascites. A neurological examination revealed paralysis of both lower limbs. Nuchal rigidity was not observed. Left inguinal region/lymph node swelling was noted. Her body temperature was 36.1°C.

Laboratory studies showed a WBC count of 8,800/μL with a neutrophil count of 89%, an RBC count of 416×10^4/μL, a hemoglobin level of 12.4 g/dL, and a blood platelet count of 2.9×10^4/μL. Her CRP level was 25.9 mg/dL, and ESR was 78 mm/h. Blood chemistry data revealed a low protein level (5.3 g/dL), a low albumin level (1.6 g/dL), and a PT-INR of 1.0. FBS and HbA1c were 173 mg/dL and 6.6%, respectively. Serum cryptococcal antigen testing was negative. QFT and HIV testing were not performed. A blood gas analysis showed a PaO_2 of 114.5 mmHg and a $PaCO_2$ of 40.9 mmHg at O_2 5 L/min through a mask. Antituberculosis agents (INH 300 mg/day, RFP 450 mg/day, ethambutol 500 mg/day, and PZA 1,200 mg/day) were immediately started.

She exhibited disseminated intravascular coagulation (DIC) on admission. Platelet transfusion, gabexate mesilate (FOY®), and human antithrombin III concentrate were also started. Transfusions of 400 mL RBCs were performed three times because of her anemia. Corticosteroid treatment was stopped for three days beginning on admission. After admission, she experienced nausea, vomiting, fatigue, dizziness, and headache. For her suspected withdrawal syndrome and nephrotic syndrome (protein urea 5.6 g/day), steroid pulse therapy (500 mg/day for three days) was also performed; subsequently, PSL was tapered (30 mg/day to 5 mg/day). Her DIC improved, and her consciousness level temporarily recovered (JCS II-20 or Glasgow Coma Scale score of five). Transiently, blood sugar control became more favorable immediately after the start of insulin injections (blood sugar: 100-200 mg/dL).

Although antituberculosis agents were administered immediately, the antimycobacterial drugs were not effective. In addition, concurrent herpes zoster and bacterial pneumonia infection occurred. Acyclovir and meropenem were additionally administered; however, the patient died of respiratory failure and heart failure (Fig. 6).

C. neoformans was cultured in lung aspiration fluid during autopsy.

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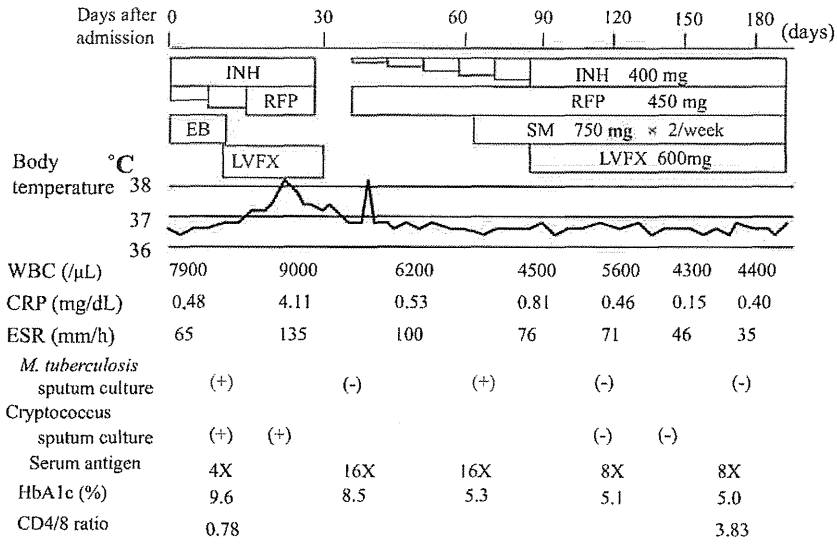

Figure 4. Clinical course of Case 2. INH: isoniazid, RFP: rifampicin, EB: ethambutol, SM: streptomycin, LVFX: levofloxacin, WBC: white blood cell, CRP: C-reactive protein, ESR: erythrocyte sedimentation rate, HbA1c: glycated hemoglobin

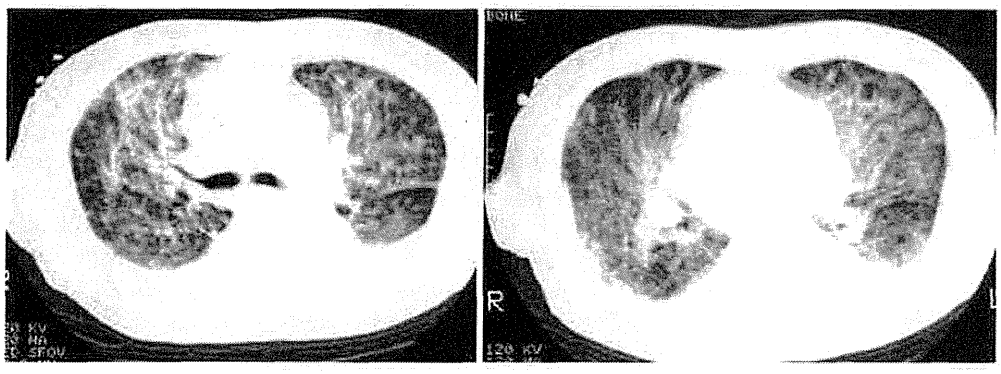

Figure 5. Thoracic CT image of case 3 on admission.

Discussion

Cases of cryptococcosis and tuberculosis co-infection are rare, even in the current HIV/acquired immunodeficiency syndrome (AIDS)-endemic era. Over the previous two decades, only a few cases of co-infection have been reported in the English literature (6-12). Between 1993 and 2006, cryptococcosis and tuberculosis co-infection was reported at one university hospital in Taiwan. That report described 23 patients with co-infection, representing 5.4% of cryptococcosis and 0.6% of tuberculosis cases. Among them, 12 patients (52%) were not infected with HIV (13). During a 35-year period in our hospital and affiliated hospitals, pulmonary cryptococcosis has been diagnosed in 151 patients. Of these, only three patients had cryptococcosis and tuberculosis co-infection (1.99%) (unpublished data).

Important underlying diseases that are common to patients with pulmonary tuberculosis and those with pulmonary cryptococcosis include immunodeficiency syndromes such as AIDS, kidney diseases, blood diseases, and cancer. In addition, the proportion of such patients who are receiving corticosteroid treatment or immunosuppressive agents is high. Diabetes mellitus is also an important underlying disease in patients with pulmonary tuberculosis and cryptococcosis.

Most studies of innate cellular immunity in patients with diabetes show decreased function (chemotaxis, phagocytosis, or killing) of polymorphonuclear cells and monocytes/macrophages, compared to controls (14). In our three patients, especially cases 1 and 2, blood sugar control was poor in the presence of other underlying diseases.

C. neoformans and *M. tuberculosis* infections are believed to be acquired through inhalation of aerosolized particles from the environment. Primary pulmonary tuberculosis is thought to be a latent infection in many cases. Pulmonary tuberculosis in elderly patients may be etiologically associated with reactivation of a latent pulmonary infection. However, the mechanism of cryptococcosis onset is still unclear. Several possibilities have been considered, including primary progression, reactivation, and reinfection (15). Persistent *C.*

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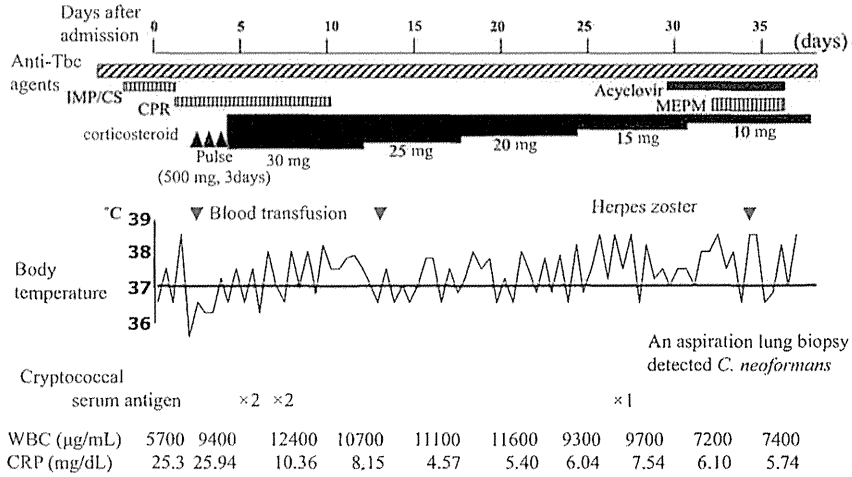

WBC (μg/mL)	5700	9400	12400	10700	11100	11600	9300	9700	7200	7400
CRP (mg/dL)	25.3	25.94	10.36	8.15	4.57	5.40	6.04	7.54	6.10	5.74

Figure 6. Clinical course of case 3. Tbc: tuberculosis, IPM/CS: imipenem/cilastatin, CPR: cefpirome, MEPM: meropenem, WBC: white blood cell, CRP: C-reactive protein

neoformans pulmonary infection is associated with intracellular parasitism (16). Moreover, recent studies support the idea that cryptococcosis onset is due to reactivation (17-19). Clinical studies have reported that patients with cryptococcosis may have developed their disease after a latent infection period of a few months to a few years (20). The finding of cryptococcal infection in two patients after ventriculoperitoneal shunting suggested reactivation of a pre-existing infection (21). These cases suggest the possibility of reactivation, as has been reported for tuberculosis.

T-cell-mediated immunity is an important defense against both mycobacterial and cryptococcal infections. It is well known that corticosteroids impair a variety of T-cell functions and inhibit the secretion of inflammatory cytokines, including interleukin (IL)-2, IL-6, IL-8, tumor necrosis factor-α (TNF-α), and granulocyte-macrophage colony-stimulating factor (22). The immunosuppressed state of case three, which was related to an increased dose of corticosteroid, may have played a central role in the development of cryptococcosis complicated by pulmonary tuberculosis.

An extremely low $CD4^+$ count (<50 cells/mm^3) is thought to be a risk factor in HIV-infected patients for the development of cryptococcosis and tuberculosis co-infection (13). However, little is known about additional risk factors for the development of co-infection in patients without HIV infection. Tuberculosis infection causes alterations in cellular immunity and is recognized as a predisposing factor for developing cryptococcosis (4, 23). Furthermore, cryptococcosis inhibits the production of TNF-α and predisposes patients to tuberculosis reactivation or infection (24, 25). Altered host immunity may explain why cryptococcosis and tuberculosis developed in these patients without HIV infection.

We did not perform HIV testing in our patients. On reflection, we should have considered doing so, even if they had evident underlying diseases and risk factors (e.g., steroid administration) that impair innate immunity and T-cell mediated immunity. HIV testing may have explained why they had mycobacterial and cryptococcal co-infection.

However, cases of cryptococcosis and tuberculosis co-infection are rare in actual clinical practice, This may be because they are rare, they may be overlooked and go undiagnosed, or cryptococcus may spontaneously resolve as in case 2.

It is known that pulmonary cryptococcosis can improve without treatment in some patients. In case 2, no antifungal agent was administered as initial therapy, due to the risk of an interaction between RFP and azole antifungal agents: RFP decreases the blood concentration and half-life of azole antifungal agents (26). Rifabutin is another option for the treatment of tuberculosis; however, the patient's clinical condition improved, making additional treatment unnecessary.

It has been reported that a central nervous system (CNS) dissemination of cryptococcal infection (cerebrospinal meningitis) develops in 14% of nonimmunosuppressed patients (27). CNS dissemination can be fatal, and it is recommended that antifungal therapy should be started immediately. In case 1, cerebrospinal meningitis developed concurrently, and AMPH-B and FLCZ were combined with antitubercular agents. The use of combination treatment with antifungal agents is controversial. Some in vitro data indicate that this combination of AMPH-B plus FLCZ may be antagonistic (28, 29); however, favorable outcomes have been described when these antifungals have been administered together, and some animal studies of the combination have shown an additive effect (30). It has been reported that combination therapy is more effective than FLCZ monotherapy (31, 32).

Treatment guidelines recommend the use of induction therapy with AMPH-B and flucytosine for cryptococcal meningitis (33). However, such treatment has not been shown to reduce mortality compared with AMPH-B alone. Recently, the results of a randomized, three-group, open-label trial of induction therapy for cryptococcal meningitis in patients with HIV infection were reported. According to

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this report, AMPH-B plus flucytosine, in comparison to AMPH-B alone, was associated with improved survival among patients with cryptococcal meningitis. Furthermore, a survival benefit for AMPH-B plus FLCZ was not found (34). On reflection, AMPH-B plus flucytosine would have been a better option for case one. However, we treated this patient before the Infectious Disease Society of America guidelines were issued and the clinical trial results were reported.

There are some commonalities in chest radiography findings in the two infectious diseases; therefore, it can be difficult to distinguish between them. The CT findings of pulmonary cryptococcosis in many cases include isolation of an area immediately below the pleura or multiple nodular shadows. Cavity formations can also be observed. However, findings vary among patients, and there is no disease-specific finding; in practice, it is often difficult to differentiate this disorder from pulmonary tuberculosis and lung cancer. In addition, these diseases sometimes show extensive consolidation, which is affected by the patient's immune state, further complicating physicians from making a definitive diagnosis. Furthermore, co-infection with cryptococcosis and tuberculosis can be difficult to distinguish clinically from cryptococcosis or tuberculosis mono-infection. Serum cryptococcal antigen test, PCR for *M. tuberculosis*, and QFT should be considered for immunocompromised patients with abnormal pulmonary shadows.

Among developed countries, Japan has a relatively high incidence of tuberculosis. The incidence of tuberculosis had been decreasing annually since the end of World War II but now shows signs of leveling off. Recent data indicate that the proportion of tuberculosis cases that occur in patients aged 65 or older has increased 1.6-fold, from 36.8% in 1987 to 59.1% in 2010; in particular, the proportion in those aged 80 or older has increased 3.8-fold, from 7.9% in 1987 to 29.7% in 2010 (35). This tendency may be associated with the phenomenon of an aging population and an increase in the number of patients with underlying diseases. In such settings, pulmonary tuberculosis remains one of the most important infectious diseases in Japan. In the future, we might encounter more cases of pulmonary tuberculosis complicated by cryptococcosis.

Author's disclosure of potential Conflicts of Interest (COI).
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