Living Donor Liver Transplantation for Patients With Hepatitis C Virus Cirrhosis: Tokyo Experience

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Living donor liver transplantation is an alternative therapeutic option for patients with end-stage HCV cirrhosis because of the cadaveric organ shortage. Preliminary results, however, indicate that live donor grafts might be disadvantageous for HCV patients. Sixty-seven patients underwent living donor liver transplantation for HCV cirrhosis between 1996 and 2004. All the patients preemptively received antiviral therapy consisting of interferon alfa-2b and ribavirin, which was started approximately 1 month after the operation. The therapy continued for 12 months after the first negative HCV RNA test. The patients were then observed without the therapy for 6 months. The therapy was continued for at least 12 months, even when the HCV RNA test remained positive. The subjects were removed from the protocol if they could not continue the therapy for 12 months because of adverse effects or could not start the therapy because of early death. Twelve patients were removed from the protocol as a result of early death (n = 9) or cessation of the drug (n = 3). Another 16 patients are currently on the protocol. Of the remaining 39 patients, 16 patients (41%) had a sustained virologic response. The cumulative 5-year survival of the HCV-positive patients was 84%, which was comparable with that of patients negative for HCV (n = 168, 86%). The present preemptive antiviral protocol after living donor liver transplantation is safe and warrants a controlled study to confirm its benefit on graft survival.

Living donor liver transplantation (LDLT) is now a common alternative procedure to deceased donor liver transplantation (DDLT), which reduces waiting-time mortality in an era of deceased donor shortage. By June 2003, 1275 LDLT cases were recorded in the European Liver Transplantation Registry.[1] The 3-year graft survival rates were 71%, although the survival rates of HCV-positive patients are unknown. In the United States,[2] 1526 adult LDLT cases were performed by May 2004. HCV is the most common indication for LDLT, and the number of HCV-positive patients is stable, approximately 100 per year between 2000 and 2002. According to the Japanese Liver Transplantation Society,[3] 1335 adult LDLT procedures were performed in Japan by the end of 2003, and of these 297 (22%) were performed for HCV cirrhosis.

A current debate in the field of liver transplantation is the possibility of increased severity of recurrent HCV infection in LDLT patients. If HCV recurs earlier and more severely after LDLT, a specific strategy for preventing the detrimental effects of HCV on living donor grafts must be developed. Preemptive interferon therapy (prophylaxis) during the early post-transplantation period might reduce the incidence and severity of HCV recurrence. In the present study, we report our results of LDLT for chronic hepatitis C and discuss the feasibility of an antiviral protocol.

Patients and Methods

We performed preemptive therapy for LDLT patients with HCV infection. From 1996–2004, 67 patients underwent LDLT for HCV cirrhosis at the Tokyo University Hospital. The patients were 51 men and 16 women, and their ages ranged from 23–63 years (median, 55 years). The HCV genotype was 1b in 53 patients (79%). Forty-one patients (61%) had hepatocellular carcinoma. Our surgical technique for recipient and donor surgery is described elsewhere.[4] All the patients received the same immunosuppressive regimens with tacrolimus (Prograf; Astellas Pharma Inc, Tokyo, Japan) and methylprednisolone as described previously.[5]

All the patients preemptively received antiviral therapy consisting of interferon alfa-2b and ribavirin, which was started approximately 1 month after the operation. The therapy was continued for 12 months after the first negative HCV RNA test. The standard regimen included interferon alfa-2b (3 million units [MU] ×3 per week) and ribavirin (800 mg/day) for 6 months. The patients were then observed without the therapy for 6 months. The therapy was continued for at least 12 months, even if the HCV RNA test remained positive.

Therapy was discontinued when there was significant leukopenia (<1500/mL), thrombocytopenia (<50,000/mL) de-

Abbreviations used in this paper: DDLT, deceased donor liver transplantation; LDLT, living donor liver transplantation.

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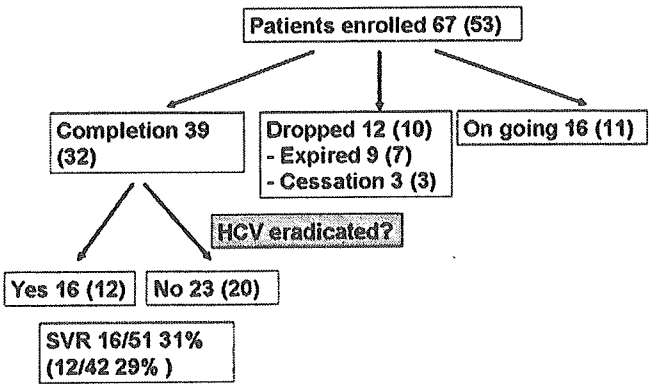

Figure 1. Results after preemptive antiviral therapy in University of Tokyo Hospital. *Numbers in parentheses* indicate those of genotype 1b. *SVR*, sustained viral response ratio. HCV eradicated? = Was the patient negative for HCV (<1000 copies/mL)?

spite application of granulocyte colony-stimulating factor (Gran; Sankyo Co Ltd, Tokyo, Japan), hemolytic anemia (hemoglobin <8 g/L), renal dysfunction (serum creatinine >2 mg/dL), depressive psychologic status, or general fatigue. The subjects were removed from the protocol if they could not continue the therapy for 12 months because of adverse effects or could not start the therapy as a result of early death.

Blood counts and liver function test results were checked every 2 weeks for the first month and at 4-week intervals thereafter. Serum samples were collected once a month for quantitative HCV RNA detection. Protocol liver biopsy was not performed. The log-rank test was used to compare the survival rate of the HCV-positive patients with the HCV-negative patients who underwent transplantation during the same period (n = 168).

Results

A total of 28 patients were excluded from the analysis (Figure 1). Twelve patients were removed from the protocol because of early death (n = 9) or because of drug cessation (n = 3). Another 16 patients are currently on the protocol and were therefore excluded from the analysis. Of the remaining 39 patients, 16 (41%) obtained a sustained virologic response. The cumulative 5-year survival of the HCV-positive patients was 84%, comparable with that of patients negative for HCV (n = 168, 86%).

Discussion

Because interferon is more effective in patients with a lower viral load,[6] initiating preemptive therapy before peak viral loads are reached is a rational approach. There is, however, a theoretical risk of increasing cellular rejection, as observed in kidney and liver transplantation.[7] Preemptive therapy during the early post-transplantation period with interferon in combination with ribavirin has been attempted in DDLT.

In a case series by Mazzaferro et al,[8] 36 recipients were treated with interferon alfa-2b (3 MU ×3 per week) and ribavirin (10 mg · kg^{-1} · day^{-1}). Treatment was started a median of 18 days after the operation and continued for 11 months. After a median follow-up of 52 months, the 5-year patient survival was 88%. Serum HCV RNA clearance was obtained in 12 patients (33%). No further antiviral treatment was required because of negative HCV RNA in serum and normal liver histology for a median of an additional 36 months. In another study,[9] 63 patients (<50% of screened cases) were randomized within 4 weeks after DDLT and treated for 48 weeks: 20 control subjects, 21 interferon alone, and 22 interferon and ribavirin. At 2 years, HCV RNA was negative in 13%, 13%, and 33%, respectively. Remarkably, there was no histologic recurrence in patients with a sustained viral response.

The association between LDLT and early HCV recurrence remains to be determined,[10] although most of the recent reports suggest that living donor graft has no effect on short-term outcome or severity of virus recurrence. Reports from New York-Presbyterian Hospital[11] indicate that the time to diagnosis of recurrent HCV is significantly shorter in LDLT. Other data indicate that the 5-year survival of HCV patients (n = 69) who undergo LDLT is 64%, which is comparable with that of DDLT patients (n = 202, 69%). The multicenter adult to adult LDLT cohort study (A2ALL) might soon provide answers to questions about recurrent HCV after LDLT and DDLT.[12]

In areas where the cavaderic organ source is almost negligible, LDLT must be selected as a therapeutic option, regardless of any potential additional risk. The results of LDLT for HCV cirrhosis in our hospital were comparable with those for non-HCV patients. If living donor graft is associated with early HCV recurrence and consequently poorer graft survival, an aggressive antiviral protocol should be performed to improve the outcome of LDLT for HCV. The present data indicate that the protocol after LDLT is safe and warrants a controlled study to confirm its benefit for graft survival.

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Clinical Transplantation

Splenectomy and preemptive interferon therapy for hepatitis C patients after living-donor liver transplantation

Kishi Y, Sugawara Y, Akamatsu N, Kaneko J, Tamura S, Kokudo N, Makuuchi M. Splenectomy and preemptive interferon therapy for hepatitis C patients after living-donor liver transplantation.
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Abstract: Recurrent hepatitis C after liver transplantation is a major cause of graft failure. We routinely perform preemptive interferon and ribavirin therapy in patients after living-donor liver transplantation indicated for hepatitis C-related cirrhosis. One of the obstacles for the therapy includes blood cytopenia. To overcome this problem, we recently performed splenectomy concurrently with liver transplantation. Thirty-five patients underwent liver transplantation and received preemptive therapy for hepatitis C. They were divided into two groups: those with splenectomy (group A, n = 21) and those without (group B, n = 14). There was no significant difference in the frequency of morbidity between the groups. Platelet counts were well maintained in group A patients during the therapy, and cytopenia led to the discontinuation of the therapy in one group B patient. The results of the preliminary study warrant a randomized control trial to examine the feasibility of splenectomy and preemptive viral therapy during liver transplantation for hepatitis C.

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Key words: hepatitis C – interferon – liver transplantation – splenectomy – thrombocytopenia

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Hepatitis C virus (HCV) infection is one of the leading etiologies for liver transplantation. The main problem of the post-transplantation course is recurrent hepatitis with 11–14% of recipients redeveloping hepatitis leading to graft failure (1, 2). However, retransplantation provides poor results, with a 3-yr survival rate of only 40–56% (3, 4).

Although interferon (IFN) and ribavirin therapy is one of the standard treatments, the sustained virologic response ratio of the therapy for recurrent HCV after transplantation is limited to approximately 30% (5–7). We routinely perform preemptive IFN therapy for recipients of living-donor liver transplantation (LDLT) indicated for HCV cirrhosis (8). One of the obstacles for starting or continuing combined IFN and ribavirin therapy includes blood cytopenia. To overcome this problem, we recently performed splenectomy concurrently with liver transplantation (9). Here we analyze the results of these patients to evaluate the feasibility of simultaneous splenectomy and combined therapy against HCV.

Patients and methods

From January 1996 to September 2004, 165 adult patients underwent LDLT. Of these, 39 recipients were indicated for HCV cirrhosis and received preemptive IFN and ribavirin therapy. Of these, four were excluded from the study because two died before the start of therapy due to uncontrolled cytomegalovirus infection or resistant acute cellular rejection, and two patients were followed up at

other hospitals and detailed laboratory data could not be obtained. The remaining 35 patients were the subjects of this study. They were divided into two groups: those with splenectomy (group A, n = 21) and those without (group B, n = 14).

The protocol of the preemptive IFN and ribavirin therapy was reported previously (8). In brief, the therapy was started when the white blood cell count was >4000 mm^3, hemoglobin level >10 g/dL, and platelet count $>100\,000$/mm^3. The therapy was initiated with 3 million units of IFN-alpha2b (Intron A; Schering-Plough K.K., Osaka, Japan) three times per week and 400 mg of ribavirin per day, which was increased up to twice the initial dose according to patient tolerance. The therapy was discontinued when there was significant leucopenia (<1500/mm^3), thrompocytopenia ($<50\,000$/mm^3) despite application of granulocyte colony-stimulating factor (G-CSF), hemolytic anemia (hemoglobin level <8 g/dL), renal dysfunction (serum creatinine >2 mg/dL) or depressive psychologic status.

Preoperative blood cell count, platelet count (mm^3), leukocyte count (mm^3), and hemoglobin (g/dL) were taken just before IFN therapy, and the numbers of days from transplantation to the start of therapy were evaluated. Blood cell counts during the therapy were examined weekly for the first month, monthly for the first year, and annually later on. The frequency of discontinuation of the therapy and its cause were reviewed. Completion of the therapy was defined as the elimination of HCV (<500 copies/mL by Amplicor HCV; Roche Molecular Systems, Pleasanton, CA, USA). Here, HCV was considered to be eliminated when the serum HCV-RNA level was consistently negative for at least 6 months after cessation of combination therapy. Protocol liver biopsy was not performed.

Data are expressed as median and range. Statistical comparison was performed using Mann–Whitney test, Fisher's exact test or repeated measure analysis of variance where appropriate. p-value <0.05 was considered statistically significant.

Results

Patient profiles

In the 17 patients of group A, the duration between LDLT and starting the therapy ranged from 18 to 59 d (Table 1). In the other four patients of group A, it was longer than 2 months as we had to wait till they recovered from pneumonia, abdominal abscess, heart failure or renal failure. The number of the patients of HCV genotype 1b (HCV_{1b}) and those of the other genotypes (HCV_{non1b}) was 5 of 16 in group A and 2 of 12 in group B. There was no significant difference in preoperative blood cell counts or liver function between the groups.

Table 1. Patients profiles

	A (n = 21)		B (n = 14)		
Group	Median	Range	Median	Range	p-value
MELD score	14	4–34	10.9	2.4–25.3	0.22
Preoperative plt ($\times10^4$/mm^3)	5.0	2.9–13.5	5.6	4.1–15.0	0.30
Preoperative WBC ($\times10^3$/mm^3)	3.3	1.3–20.5	2.8	1.6–9.8	0.51
Preoperative Hb (g/dL)	9.0	5.5–12.7	10.5	5.6–13.3	0.24
Start day (d)	41	18–120	30	7–130	0.34
HCV-RNA before therapy (kcopies/mL)	663	186–3350	510	46–1700	0.66

MELD, model for end-stage liver disease; plt, platelet; WBC, white blood cell; Hb, hemoglobin.

Postoperative infectious diseases

In group A, six (29%) patients suffered from infectious disease: four from abdominal abscess, one from fungal pneumonia and one from bacterial pneumonia. Two of the four abdominal abscesses were related to the splenectomy because there was pancreatic juice leakage from the drainage tube in the left subphrenic space. Both of the patients responded well to surgical re-exploration. In group B, five (36%) patients had infection episode with no mortality including three abdominal abscesses, one sepsis and one osteomyelitis.

Blood cell counts after interferon and ribavirin therapy

In group A patients, platelet count significantly increased soon after LDLT and was maintained during the treatment for up to 2 yr (Fig. 1). Platelet count was kept higher in group A patients ($p = 0.008$) during the observation period. Leukocytopenia (<3000/mm^3) were observed in three patients of group A and seven in group B. All of them were well controlled by G-CSF except for one in group B who discontinued the therapy because of cytopenia.

Continuation of therapy

Six (29%) patients in group A and three (21%) in group B discontinued therapy before the HCV was eradicated (Table 2). A 40-yr-old male in group A underwent retransplantation for cholestatic

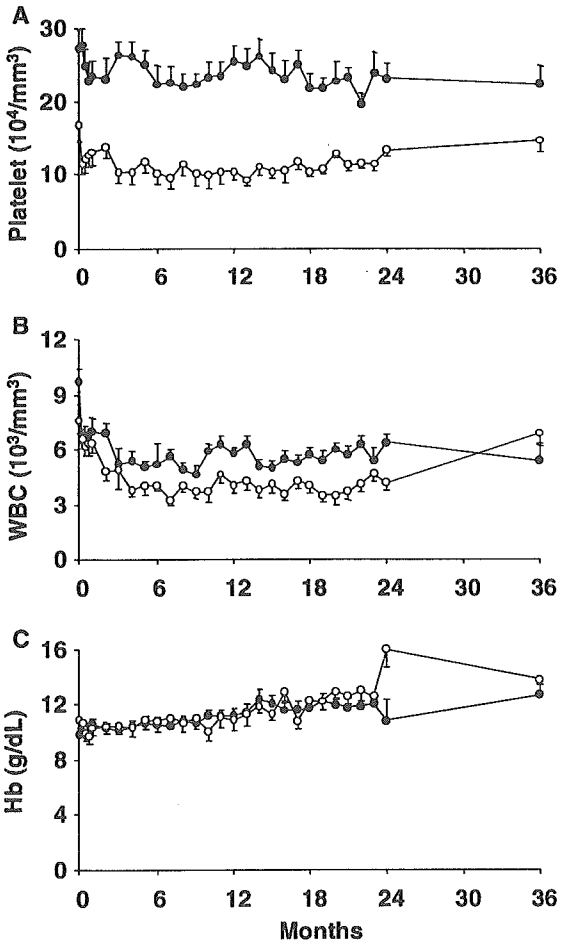

Fig. 1. Changes of platelet (A), white blood cell count (B) and hemoglobin (C). Levels after interferon therapy in group A (thick line with close circles) and group B (thin line with open circles). The bar represents a standard error value. There was a significant difference between the groups in the platelet count ($p = 0.008$).

Table 2. Timing [months after the start of interferon (IFN) therapy] and the reason of cessation of IFN therapy

Group	Patient	Timing	Reason
A	1	14	Renal dysfunction
	2	7	Depression
	3	7	Death caused by thrombotic thrombocytopenia
	4	18	Retransplantation because of cholestatic hepatitis
	5	19	Renal dysfunction
	6	3	Depression
B	1	4	Death caused by virus associated hemophagocytotic syndrome
	2	9	Thrombocytopenia
	3	6	Death because of hepatocellular carcinoma recurrence

hepatitis 18 months after the primary LDLT and died of liver failure 4 months after the retransplantation. Four patients in group A and three in group B completed the therapy. Eleven patients in group A and eight in group B continued the therapy for 21 (range: 11–47) and 24 (range: 11–66) months, respectively.

Effect of genotype

In group A, HCV-RNA became negative in 44% (7/16) of HCV_{1b} patients and 60% (3/5) of the HCV_{non1b}. Median periods of treatment until the RNA level became negative was 15 (range: 1–18) months and 2 (range: 2–8) months in each group, respectively. There was no significant difference in the period by genotype ($p = 0.30$). In group B, HCV-RNA became negative in 17% (2/12) of HCV_{1b}, and 100% (2/2) of HCV_{non1b}.

Discussion

Preemptive IFN and ribavirin therapy to prevent cholestatic hepatitis has not been established. Only a few centers, including ours, report using this strategy (8, 10–13). Among the 39 patients who underwent preemptive IFN therapy after liver transplantation with or without splenectomy, we experienced cholestatic hepatitis in only one patient, which might indicate the possibility that long-term IFN and ribavirin therapy prevents the occurrence of cholestatic hepatitis. Gopal and Rosen (14) reported the results of IFN and ribavirin therapy in seven cholestatic hepatitis patients with only two patients who survived for an average of 32 months. They emphasized the importance of continuing the therapy indefinitely because the cessation of the therapy even after 12 months or more of treatment with sustained HCV-RNA negativity led to rapid recurrence of cholestatic hepatitis. IFN and ribavirin therapy might be worth continuing over the long term, especially in patients with HCV_{1b}. The preemptive therapy is effective in cases with lower HCV-RNA levels and less graft injury by the virus (11, 13). Accordingly, the treatment should be started within a short interval of transplantation.

The indications for simultaneous splenectomy in liver transplantation for reducing portal hypertension to protect the graft from congestion, especially in small left liver graft, or repairing portal flow regurgitation are established (15, 16). The effectiveness of splenectomy against thrombocytopenia is reported (9, 17). Several authors, however, have objected to perform splenectomy as a therapeutic option for thrombocytopenia because it might increase the risk of septic complications postoperatively, and instead recommend splenic artery ligation or radiologic partial splenic embolization (18–21). Several reports, however, suggest that

the indication of such ligation or embolization methods should also be considered with care because of the low success rate and risk of complications (22, 23). We previously reported the safety of concomitant splenectomy and several other centers report similar good results (9, 24). The results of the present study suggest that splenectomy is feasible for starting combination therapy early after transplantation and continuing for up to 4 yr with an acceptable morbidity rate.

The long-term effect of splenectomy as a therapeutic option for blood cytopenia because of portal hypertension remains unclear in patients undergoing IFN and ribavirin therapy. Randomized control trials to examine the risk and benefits of splenectomy for patients undergoing liver transplantation and combined therapy for hepatitis C are necessary.

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Philip A. Mackowiak, Section Editor

Bilateral Lesions in the Basal Ganglia of a Patient with Acquired Immunodeficiency Syndrome

(See page 943 for Photo Quiz)

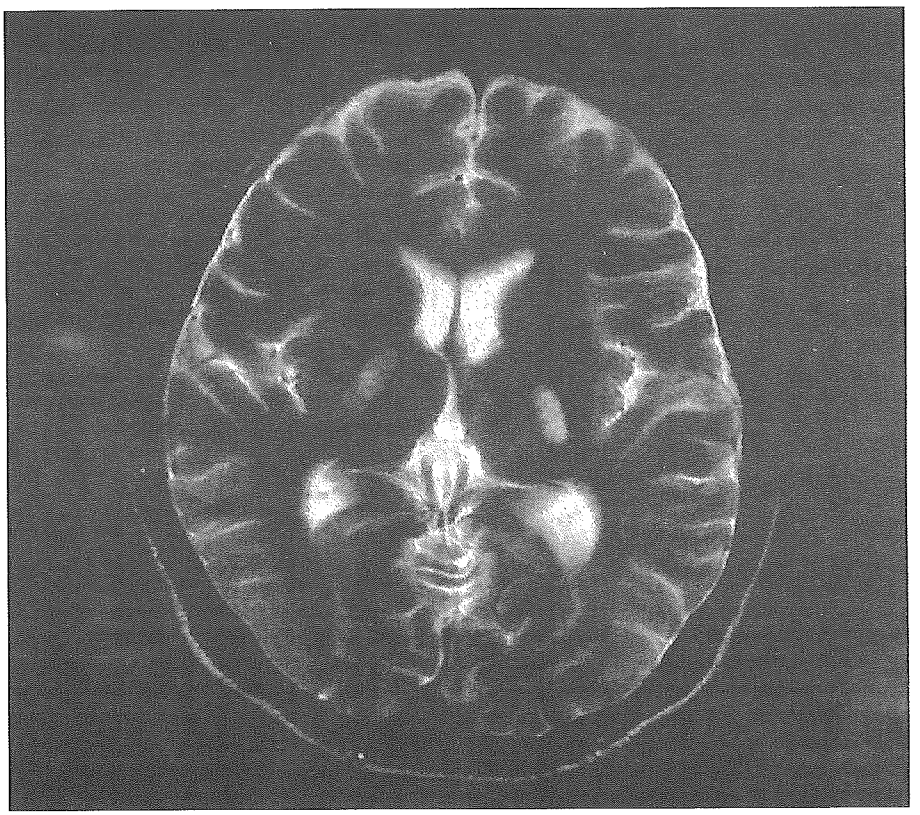

Figure 1. T2-weighted MRI of the brain of a 41-year-old man with AIDS demonstrating bilateral hyperintense lesions in the basal ganglia *(arrows)*

Diagnosis: Cryptococcal gelatinous pseudocysts with meningitis.

In patients with AIDS who have cerebral mass lesions, diagnoses of toxoplasmosis and lymphoma should always be considered. However, these lesions usually show contrast enhancement and edema [1], which was not the case for our patient. Furthermore, our patient was *Toxoplasma* seronegative, and the results of PCR of CSF specimens for *Toxoplasma* DNA were also negative [2]. Brain thallium-201 single-photon emission CT did not reveal abnormal accumulation in these lesions [3]. If these findings are taken together, cerebral toxoplasmosis and lymphoma are not likely diagnoses.

Cryptococcosis in the CNS is the third-most frequent neurological complication in patients with AIDS [4], and it usually presents as sole meningitis without cerebral focal lesions [5]. However, cerebral focal lesions can also develop [6]. These

lesions are divided into 4 groups on the basis of MRI pattern: (1) parenchymal mass lesions, (2) gelatinous pseudocysts, (3) multiple miliary enhancing parenchyma and leptomeningeal-cisternal nodules, and (4) a mixed pattern [7]. Gelatinous pseudocysts are dilated perivascular spaces around perforating blood vessels (Virchow-Robin spaces) filled with cryptococci and their mucoid exudates, which are hypointense on T1-weighted images and hyperintense on T2-weighted images [7, 8]. Gelatinous pseudocysts usually occur symmetrically in the basal ganglia (corresponding to the distribution of the perforating arteries) and are round or oval in shape. Because the blood-brain barrier remains intact, no contrast enhancement is seen on either CT scans or MRIs.

In conclusion, in our patient, the MRI pattern was typical of cryptococcal gelatinous pseudocysts (figure 1). India ink staining of a CSF specimen revealed *Cryptococcus* species, and culture of the specimen confirmed it was *Cryptococcus neoformans*. A 2-week course of amphotericin B lipid complex [9] partially resolved the paralysis of his extremities and weakened the intensity of the cerebral lesions on MRI. CSF culture results became negative, and maintenance treatment with fluconazole was administered.

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Dilated Cardiomyopathy in an Adult Human Immunodeficiency Virus Type 1–Positive Patient Treated with a Zidovudine-Containing Antiretroviral Regimen

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We describe an adult woman infected with human immunodeficiency virus type 1 (HIV-1) who developed dilated cardiomyopathy (DCM) with histologically confirmed mitochondrial damage while receiving anti–HIV-1 combination therapy that included nelfinavir, lamivudine, and zidovudine. DCM resolved after discontinuation of the regimen, and cardiac function remained normal after initiation of treatment with nelfinavir, lamivudine, and abacavir, which indicates that DCM was induced by mitochondrial toxicity, most likely caused by zidovudine.

Nucleoside reverse-transcriptase inhibitors (NRTIs) can cause mitochondrial toxicity that manifests as dysfunction of various organs [1]. We report a case of dilated cardiomyopathy probably induced by zidovudine.

Case report. A 36-year-old woman who acquired HIV-1 infection through sexual intercourse was initially treated in March 1999 with a HAART regimen that included stavudine, lamivudine, and nelfinavir. The patient's baseline CD4 cell count and plasma HIV-1 RNA load were 231 cells/μL and 8.2×10^4 copies/mL, respectively. Her virus load was successfully suppressed to a level below the limit of detection (50 copies/mL). However, stavudine was replaced with zidovudine in February 2000 because of peripheral neuropathy. The patient's CD4 cell count had been >300 cells/μL and her virus load had been below the limit of detection since August 1999. The patient had no history of opportunistic infections or cardiovascular disease.

In October 2001, 20 months after the commencement of the zidovudine-containing regimen, the patient complained of shortness of breath. In March 2002, she reported worsening of shortness of breath, coupled with nonproductive cough, particularly at night. She was admitted to the hospital on 4 April 2002 for evaluation of the cause of those symptoms. On admission, her body temperature was 38°C, her blood pressure was 190/130 mm Hg, and her pulse rate was 116 beats/min. Arterial oxygen saturation, measured noninvasively by pulse oximetry, was 99% during room air breathing. Physical examination showed pretibial edema and audible third and fourth heart sounds. Chest radiography revealed cardiomegaly and pulmonary vascular redistribution. Electrocardiography showed sinus tachycardia and nonspecific ST-T changes. On echocardiography, dilation of the left ventricle with a low left ventricular ejection fraction of 35% and diffuse left ventricular hypokinesia were observed. The CD4 cell count on admission was 630 cells/μL, and the virus load was below the limit of detection. Laboratory data on admission showed elevated levels of liver enzymes (aspartate transferase, 67 IU/L; alanine transferase, 98 IU/L), creatine kinase (89 IU/L; creatine kinase–MB, 8 IU/L), and lactate (2.6 mmol/L). Blood bicarbonate levels (36.9 mm Hg) and serum lipid levels (cholesterol, 219 mg/dL; triglycerides, 119 mg/dL) were within normal limits. There was no clinical evidence of lipodystrophy. Losartan potassium, carvedilol, furosemide, and spironolactone were administered for management of the congestive heart failure.

At first, infectious myocarditis was considered the likely cause of congestive heart failure in this patient because of the presence of diffuse hypokinesia of the heart accompanied by fever. However, the results of serological tests for cardiotropic viruses (coxsackievirus, adenovirus, herpes simplex virus, respiratory syncytial virus, and Epstein-Barr virus), *Toxoplasma gondii, Chlamydia trachomatis, Chlamydia psittaci,* and *Chlamydia pneumoniae* were all negative. The results of blood cultures and of tests for cytomegalovirus antigenemia were also negative. We also suspected cardiomyopathy induced by zidovudine, because high lactate levels sometimes reflect mitochondrial toxicity caused by NRTIs [2], and the patient did not use any other drugs associated with cardiac toxicity. Thus, HAART was discontinued on admission to the hospital.

Cardiac catheterization performed 11 days after admission revealed a normal coronary artery. A biopsy specimen from the right ventricle was obtained. Histopathological exami-

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nation of the specimen showed no infiltration of inflammatory cells but slightly hypertrophied cardiac myocytes with mild interstitial fibrosis (figure 1). Electron microscopic examination revealed inequality of muscle fibers, degenerated cardiac myocytes, accumulation of glycogen particles, and severely altered mitochondriosis and intramitochondrial myelin-like figures (figure 2).

The patient's clinical condition gradually improved after discontinuation of HAART. Cardiomegaly and pulmonary vascular redistribution disappeared, and the ejection fraction was 45% at 3 months after the discontinuation of the zidovudine-containing regimen. A new HAART regimen that included lamivudine, abacavir, and nelfinavir was initiated on 8 December 2002; as of March 2003, no clinical signs of congestive heart failure had been noted.

Discussion. This case of histopathologically confirmed dilated cardiomyopathy was probably caused by zidovudine. The reasons for suspecting that zidovudine was the cause of the cardiac pathologic findings are as follows: (1) the clinical symptoms appeared after the commencement of HAART that included zidovudine, (2) the clinical signs and symptoms gradually improved 3 months after discontinuation of the zidovudine-containing HAART regimen, and (3) cardiac function remained normal after the introduction of new HAART regimen that included lamivudine, abacavir, and nelfinavir. Previous stavudine treatment, which had been administered for 11 months before the change to zidovudine, might have contributed to the development of the cardiomyopathy. However, the clinical symptoms appeared 20 months after the switch from stavudine to zidovudine. Therefore, zidovudine seemed to have played a key role in the pathogenesis of this case.

Several reports have indicated that zidovudine can induce cardiomyopathy in animals [3] and in children [4]. Recently, Frerichs et al. [5] reported the first adult case of cardiomyopathy caused by a zidovudine-containing therapeutic regimen. However, this report did not identify the causative drug, because the patient was treated with a 3-drug combination regimen that included zidovudine. The association of cardiomyopathy with zidovudine in adults is still unclear. The mechanism of zidovudine-induced cardiomyopathy is thought to be mitochondrial toxicity caused by zidovudine, made evident by depletion of mitochondrial DNA levels [2]. In our case,

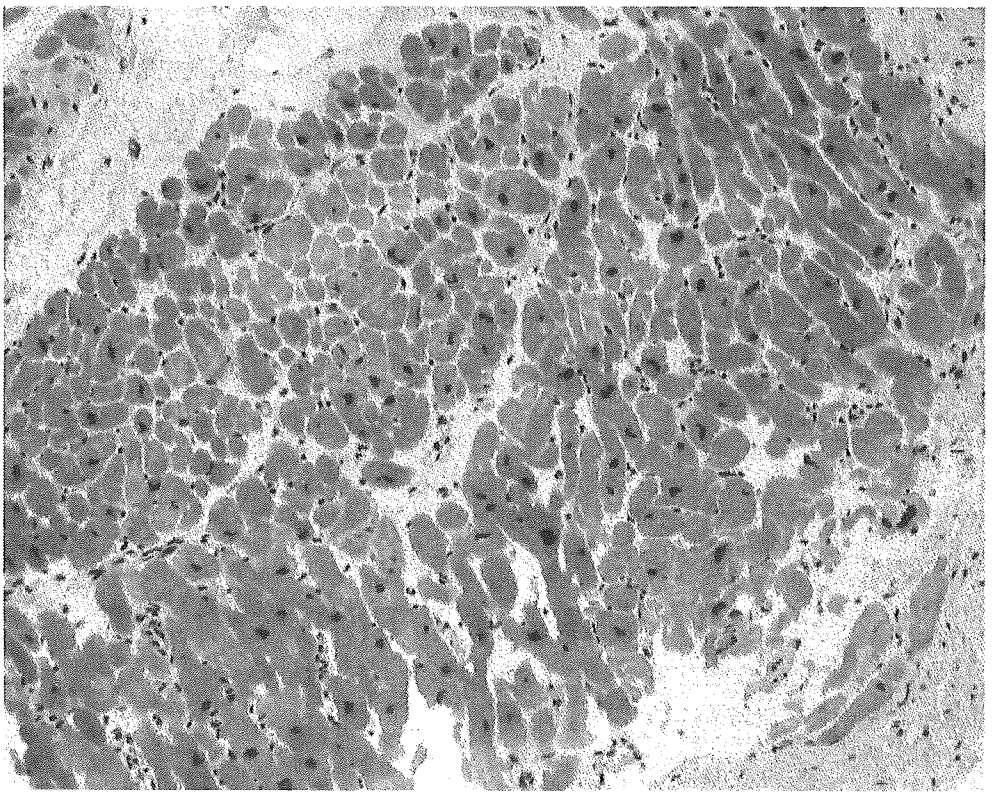

Figure 1. Micrograph of a myocardial biopsy specimen. Note the lack of inflammatory cells. No infiltration of inflammatory cells is seen, but slightly hypertrophied cardiac myocytes with mild interstitial fibrosis are present. (Hematoxylin-eosin; original magnification, ×100.)

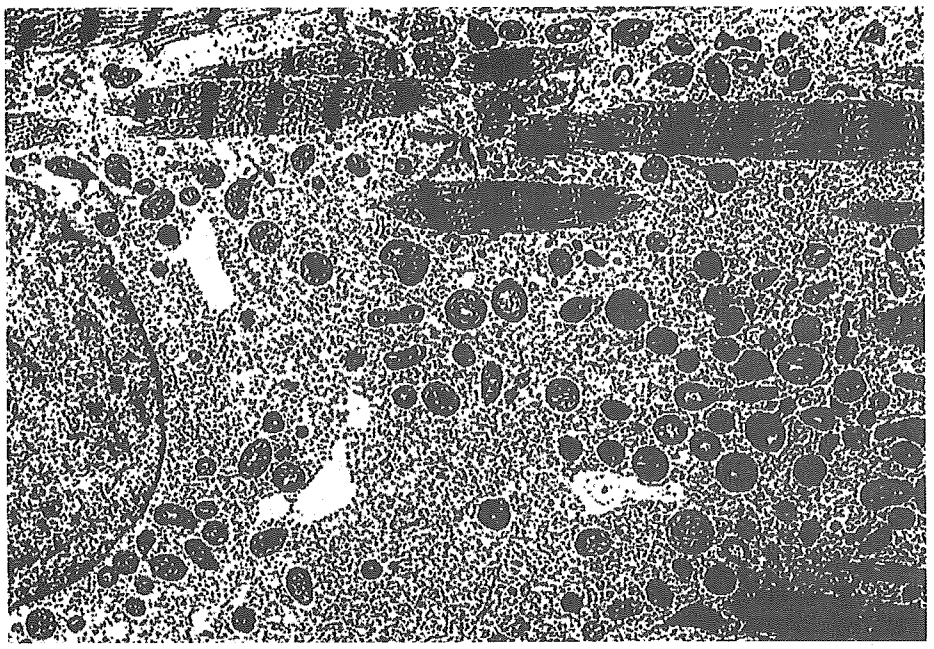

Figure 2. Electron micrograph of a myocardial biopsy specimen showing inequality of muscle fibers, degenerated cardiac myocytes, accumulation of glycogen particles, and severely altered mitochondriosis and intramitochondrial myelin-like figures. (Original magnification, ×25,000.)

we also found severely altered mitochondria on an electron micrograph. These changes and the high concentration of lactate suggested mitochondrial damage, probably induced by zidovudine. Physicians should be aware of the possibility of zidovudine-induced cardiac toxicity.

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Primary nelfinavir (NFV)-associated resistance mutations during a follow-up period of 108 weeks in protease inhibitor naïve patients treated with NFV-containing regimens in an HIV clinic cohort☆

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Abstract

Background: Nelfinavir (NFV) is a widely prescribed HIV-1 specific protease inhibitor (PI). However, there are only a few reports that have described the long-term effects of NFV-containing regimens, especially with regard to the emergence of drug resistance in inner-city clinics. *Objectives:* The aim of this study was to investigate the clinical and virologic responses to treatment with NFV-containing regimens for up to 108 weeks and determine the timing and rate of emergence of primary NFV-resistance associated mutations in daily clinical practice. *Study design:* A cohort study in an inner-city clinic. Our study included 51 consecutive patients who were PI-naïve and commenced therapy in February 1997 through April 1999. *Results and conclusions:* The proportions of patients who continued the same therapeutic regimen and showed virologic success (viral load < 400 copies/ml) up to 108 weeks were 78 and 63%, respectively, based on intent-to-treat analysis. Among patients with a viral load persistently > 400 copies/ml at week 12 ($n = 30$), 11 developed primary NFV-resistance associated mutations by 108 weeks (stratified log-rank test; $P < 0.05$). The Cox proportional hazard model showed that prior use of reverse transcriptase inhibitors ($n = 22$) (relative hazard (RH); 2.10, 95% CI; 0.67–6.62), prior AIDS diagnosis ($n = 6$) (RH; 1.70, 95% CI; 0.37–7.77), CD4 $< 200/\mu l$ at baseline ($n = 19$) (RH; 2.48, 95% CI; 0.78–7.81) and viral load $> 30{,}000$ copies/ml at baseline ($n = 21$) (RH; 2.10, 95% CI; 0.67–6.62) were not independent predictors of the NFV-resistance, although some tendency was noted. In total, 77% of the patients continued NFV-containing treatment without the NFV-resistance for 108 weeks. The viral load at week 12 could be used as a predictor of treatment success in our cohort study.

Keywords: HIV-1; Nelfinavir; Drug resistance; Cohort study; Inner-city clinic

☆ The accession numbers of the nucleotide sequence were assigned at the DDBJ as follows: AB020911–020925.

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1. Introduction

Morbidity and mortality related to HIV-1 infection have markedly diminished in those countries in which highly active antiretroviral therapy (HAART) is available (Egger et al., 1997; Hammer et al., 1997; Palella et al., 1998; Murphy et al., 2001). Among the six HIV-1-specific protease inhibitors (PI) approved as of 2001, nelfinavir (NFV) is often prescribed because of its very active antiviral and clinical efficacy (Easterbrook et al., 2001). Therefore, NFV is frequently used as the control drug in many clinical trials of investigational drugs (Podzamczer et al., 2001; Ruane et al., 2001), as well as salvage therapy in those patients in whom initial therapies had failed (Seminari et al., 1999; Roca et al., 2000; Albrecht et al., 2001). However, there are only a few reports that have described the long-term effects of NFV-containing regimens (Gathe et al., 2000), especially with regard to the emergence of drug resistance in inner-city clinics.

In clinical trials, the selection of participants is not only based on specific inclusion criteria of trials, but also on adherence to the designed regimens to achieve maximum clinical effect (Paterson et al., 2000). Therefore, virologic success (viral load <400 copies/ml) has been described in such trials to occur in 70–90% of patients (Hammer et al., 1997; Markowitz et al., 1998; Ruane et al., 2001). However, in daily clinical practice, patients form a heterogeneous group of individuals with various demographic, behavioral and clinical features. Therefore, the clinical effects of HAART in such situations have been reported to be considerably less successful compared with clinical trials (Fatkenheuer et al., 1997; Lucas et al., 1999; Mocroft et al., 2000).

In the absence of suppression of viral load during treatment with NFV-containing regimens, selection and accumulation of NFV-associated resistance mutations is inevitable (Patick et al., 1998; Tebas et al., 1999). The majority of the initial substitutions emerged in NFV-containing regimens and included aspartic acid (D) to asparagines (N) substitution at residue 30 (D30N) and/or from leucine (L) to methionine (M) at residue 90 (L90M) of the PR gene (primary mutation of NFV resistance) (Hirsch et al., 2000). In this regard, the L90M mutation causes cross-resistance to saquinavir (SQV) (Hirsch et al., 2000), which reduces the clinical effects of subsequent regimens including SQV (Gatanaga et al., 1999; Tebas et al., 1999).

The aim of the present study was to investigate the timing and rate of emergence of NFV-resistance in clinical practice. For this purpose, we retrospectively investigated the clinical and virologic outcomes in PI-naïve otherwise unselected patients treated with NFV-containing regimens for up to 108 weeks.

2. Materials and methods

2.1. Study design and patients

Consecutive patients who were PI-naïve and commenced treatment containing NFV between February 1997 and April 1999 at the AIDS Clinical Center, International Medical Center of Japan were included in this study. Patients visited our clinic once a month where, in addition to clinical examination, CD4 counts and viral load were determined. Before starting any anti-HIV regimens, we extensively provided all patients with detailed information regarding the importance of treatment, the method of taking many pills, possible adverse events and strategies to deal with such effects and finally, the importance of full adherence to treatment and regular visits by doctors and coordinator nurses. In our clinic, almost all patients agreed to participate in retrospective clinical studies and serum stocks from residues of routine examinations of blood chemistry for such future studies were maintained after obtaining a signed informed consent. The institutional ethics committee approved this study in August 2001. Then, a retrospective analysis of the medical records by the end of May 2001 was completed in August 2001. Thus, the follow-up period of this study was 108 weeks. Most patients were treated with 1250 mg NFV twice daily, combined with two reverse transcriptase inhibitors (RTI). Adherence and adverse events were recorded at each visit. Adverse events were graded

according to the rating scale of the ACTG (Division of AIDS, 1996).

2.2. Measurement of viral load and CD4 count

Plasma viral load was measured in our hospital by using the Roche Amplicor assay kit (Roche Diagnostic Systems, Branchburg, NJ) version 1.0 by September 1999 and version 1.5 thereafter. Since the detection limit of these two kits is different, any viral load recorded as <400 copies/ml was considered undetectable viral load was transformed to $\log_{10}$ values. CD4 count was analyzed using standard flow cytometry techniques.

2.3. Sequence analysis

Sera were stored at −80 °C. Sequence analysis of the protease gene was performed using the method described previously (Gatanaga et al., 1999). Total RNA was extracted from 80 μl of serum by the SMITEST EX-R&D (Genome Science Laboratories, Fukushima, Japan) and the pellet was resuspended in 25 μl of RNA-free water. The RNA was reverse transcribed at 50 °C for 30 min and subjected to the first polymerase chain reaction (PCR) with primers DRPO1 (sense) and DRPO2 (antisense) using one-step RNA PCR Kit (TaKaRa, Kyoto, Japan) followed by the second PCR with primers DRPO3 (sense) and DRPO4 (antisense). Each procedure consisted of 30 cycles of denaturing at 94 °C for 30 s, annealing at 50 °C for 30 s and extension at 72 °C for 30 s. Primer sequences of DRPO1, 2, 3 and 4 were as follows, respectively: DRPO1, 5′-CCAACAGCCCCACCAGA-3′ (MN *pol* positions, 2152–2168), DRPO2, 5′-ATTTTCAGGCCCATTT TTTGA-3′ (MN *pol* positions, 2711–2691), DRPO3, 5′-AGCAGGAGACGATAGACAAGG-3′ (MN *pol* positions, 2213–2233) and DRPO4, 5′-CTGGCTTTAATTTTACTGGTA-3′ (MN *pol* positions, 2592–2572). PCR products were directly submitted to sequence analysis using an automatic sequencer (model 377, Applied Biosystems, Foster City, CA) and the Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) using the conditions recommended by the supplier. The nucleotide sequence was translated to the amino acid sequence by the GENETYX-WIN version 4.0 (Software Development, Tokyo, Japan). Sequence data were compared to the HIV-1 clade B consensus sequence.

2.4. Definition of virologic success and time of sequence analysis

Suppression of the viral load to <400 copies/ml at 12 weeks after the commencement of treatment was considered as successful treatment (virological success) and the causative virus was considered free of primary mutations. All sera at baseline were sequenced to confirm that there were no primary NFV resistance-associated mutations (D30N and/or L90M). If viral load was >400 copies/ml at 8 weeks after the commencement of treatment, a genotypic resistance assay was performed at that stage and every 4 months thereafter until the primary mutations were detected.

2.5. Statistical analysis

CD4 count and viral load were recorded every month. Analyses were censored every 4 months. If CD4 count and viral load were not available at the censored time, data of 1 month earlier were used. Primary efficacy was assessed based on virologic success. Rate of virologic success was analyzed by the intent-to-treat principle. The time to various outcomes, such as time to virologic success, time to discontinuation of treatment and time to emergence of NFV-resistance mutations, were estimated using the Kaplan–Meier analysis and compared using the stratified log-rank test. The Cox proportional hazard model was used to estimate event rate ratios with 95% CI for potential predictors of emergence of resistance mutations. These included prior AIDS diagnosis, prior RTI therapy, CD4 <200/μl at baseline and viral load over 30,000 copies/ml at baseline. The Wilcoxon signed-rank test was used to assess changes in CD4 and viral load after treatment. All reported P values are two-tailed and $P<0.05$ was considered significant. Analyses were performed using StatView software package version 5.0 (SAS Institute, Cary, NC).

3. Results

3.1. Baseline characteristics

We identified 51 patients who were PI-naïve, commenced treatment with NFV-containing regimens and followed their clinical courses for at least 108 weeks as of May 2001. No other selection criteria were used to enroll patients in this study. Table 1 shows the baseline characteristics of these patients. CD4 count at baseline varied from 1 to 680/μl (median; 237/μl) and viral load also varied from undetectable (<400 copies/ml) to 6.4 $\log_{10}$ copies/ml (median: 4.3 $\log_{10}$ copies/ml). Six patients (12%) had histories of AIDS-related illnesses before commencement of NFV therapy. Twenty-two patients (43%) had used RTI in the past. Thus, our population sample was very heterogeneous with regard to CD4 count and viral load at baseline, prior RTI therapy and prior AIDS diagnosis, but all patients were PI-naïve and the majority were Japanese.

Table 1
Baseline characteristics of participating patients

Parameter	Patients ($n = 51$)
Mean age in years (range)	36 (20–71)
Males *n* (%)	45 (88)
Ethnic group n (%)	
Japanese	48 (94)
Asian other than Japanese	2 (4)
Hispanic	1 (2)
Route of transmission n (%)	
Male homosexuals	31 (60)
Heterosexual	10 (20)
Hemophilia	10 (20)
CD4 count, median (range) cells/μl	237 (1–680)
Plasma viral load, median (range) log $_{10}$ copies/ml	4.3 (undetectable–6.4)
Previous AIDS defining illnesses *n* (%)	6 (12)
RTI-experienced *n* (%)	22 (43)
Drugs combined with nelfinavir n (%)	
Stavudine (d4T)+lamivudine (3TC)	21 (41)
Zidovudine (AZT)+3TC	18 (35)
AZT+zalcitabine (ddC)	7 (14)
D4T+didanosine (ddI)	3 (6)
AZT+ddI	2 (4)

RTI, reverse transcriptase inhibitor.

3.2. Continuation of original treatment regimens

All patients survived at week 108. Estimated probabilities of continuation of the original regimen at weeks 48 and 108 were 86% (95% CI; 76.8–95.7%) and 78% (95% CI; 67.1–89.7%), respectively (Fig. 1). During the course of follow-up, 11 patients changed their original regimens (Table 2). Among them, five discontinued their regimens due to grade III adverse events (including four who developed generalized drug eruption within 2 weeks of commencement of therapy and one developed liver toxicity at week 76) and six patients changed their regimens because of virologic failure, all of whom harbored viruses with resistance mutations before failure.

3.3. CD4 count and virologic responses

Fig. 2 shows the mean increase in CD4 count from baseline. The mean increase of CD4 count (95% CI) at weeks 24, 48, 72 and 108 were 93/μl (61–124/μl), 129/μl (94–164/μl), 168/μl (120–215/μl) and 166/μl (116–217/μl), respectively. At each time point, the mean increase in CD4 count from baseline was significant ($P < 0.05$). The rate of virologic success (viral load <400 copies/ml) at week 108 was 63% by intent-to-treat analysis (Fig. 3). The median decrease $\pm$S.D. of viral load at weeks 24, 48, 72 and 108 from baseline were -1.38 ± 0.84, -1.54 ± 0.91, -1.49 ± 0.91 and -1.51 ± 0.99 $\log_{10}$ copies/ml, respectively. The decrease in viral load from baseline was significant ($P < 0.05$) at each time point.

3.4. Emergence of resistant mutations

Time to emergence of primary NFV-resistance mutations, D30N and/or L90M, is shown in Fig. 4(A). The earliest emergence at D30N was 9 weeks after commencement of therapy and at L90M was 48 weeks. In three cases, L90M was added to D30N harboring mutants. In total, 12 of 51 patients (24%) had D30N and/or L90M substitutions up to 108 weeks. When virologic success was

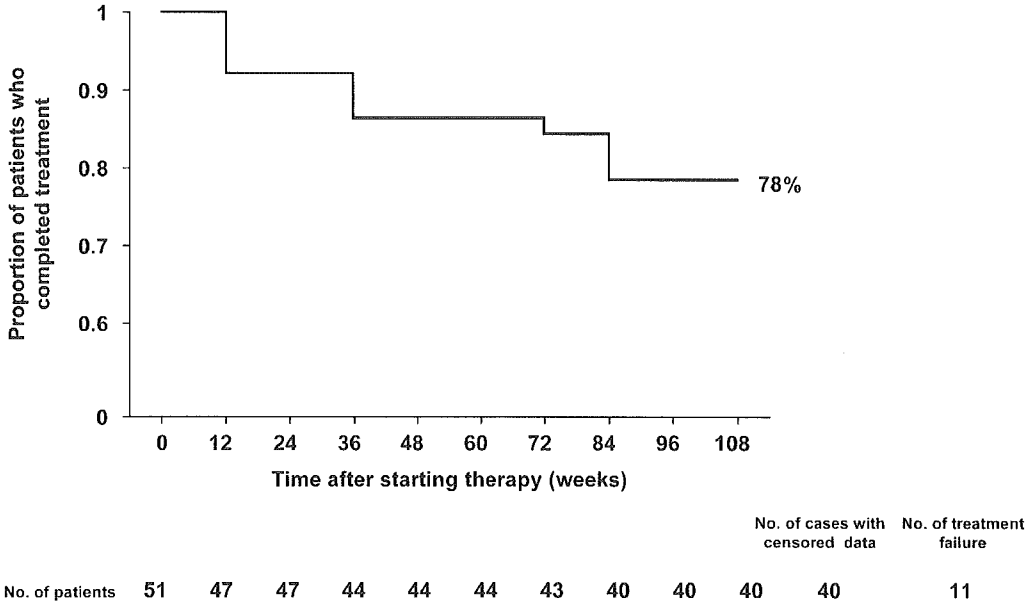


Fig. 1. Kaplan–Meier estimates of the time to discontinuation of nelfinavir-containing regimens.

established at week 12, all patients except one (16 of 17 patients) continued the same regimen up to 108 weeks without any primary resistance mutations. In contrast, the viral load of 30 patients remained >400 copies/ml at week 12 (four patients had already discontinued their original regimens due to drug-related eruption by week 2; Table 2). Among them, 11 had resistance mutations by 108 weeks (the stratified log-rank test; $P < 0.05$) (Fig. 4B) and seven patients changed their regimens due to treatment failure. When patients were stratified into the antiretroviral-naïve ($n = 29$) (ART-naïve) and PI-naïve but RTI-experienced ($n = 22$), the resistance mutations tended to appear earlier in the RTI-experienced group than in the ART-naïve group (Fig. 4C).

Table 2
Patients who discontinued NFV-containing therapeutic regimen

Pt	Original regimen*	Discontinuation of original regimen		Replacement regimen	Resistant mutations (detected at week)
		At week	Reason		
1	AZT/ddI	2	Drug eruption	AZT/ddI/SQV	None
2	AZT/3TC	2	Drug eruption	No therapy	None
3	AZT/3TC	2	Drug eruption	AZT/3TC/SQV	None
4	d4T/3TC	2	Drug eruption	No therapy	None
5	d4T/ddI	76	Liver toxicity	No therapy	None
6	AZT/ddC	73	Virologic failure	D4T/3TC/RTV/SQV	D30N (16)
7	d4T/ddI	25	Virologic failure	d4T/ddI/RTV/SQV	D30N (12)
8	AZT/3TC	63	Virologic failure	d4T/3TC/RTV/SQV	D30N (47)
9	AZT/3TC	28	Virologic failure	d4T/3TC/RTV/SQV	D30N (16)
10	d4T/3TC	34	Virologic failure	d4T/ddI/IDV	D30N (9)
11	d4T/3TC	78	Virologic failure	AZT/ddI/APV	L90M (76)

AZT, zidovudine; ddI, didanosine; ddC, zalcitabine; d4T, stavudine; 3TC, lamividine; NFV, nelfinavir; IDV, indinavir; SQV, saquinavir; RTV, ritonavir; APV, amprenavir; D, aspartic acid; N, asparagine; L; leucine; M, methionine.
* Combined with NFV.

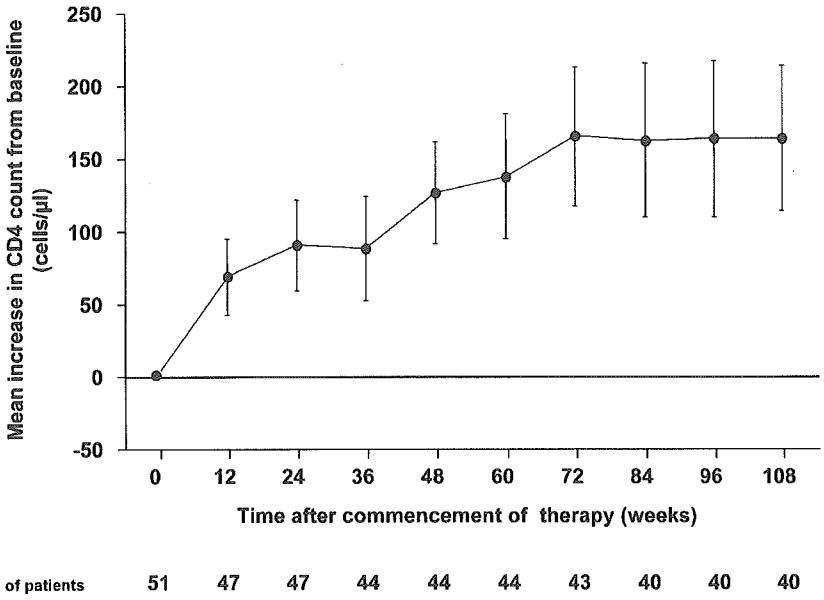


Fig. 2. Mean changes in CD4 count from baseline. Vertical bars represent the 95% confidence interval.

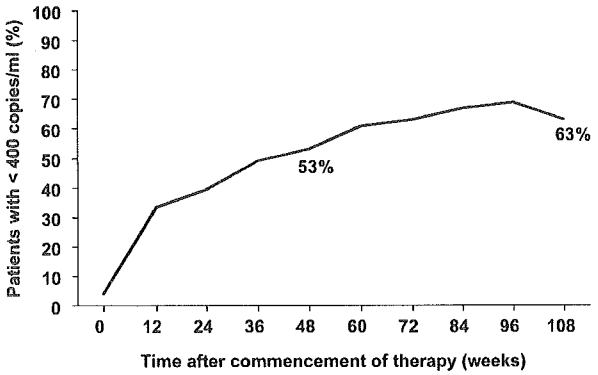


Fig. 3. Percentage of patients with plasma viral load <400 copies/ml.

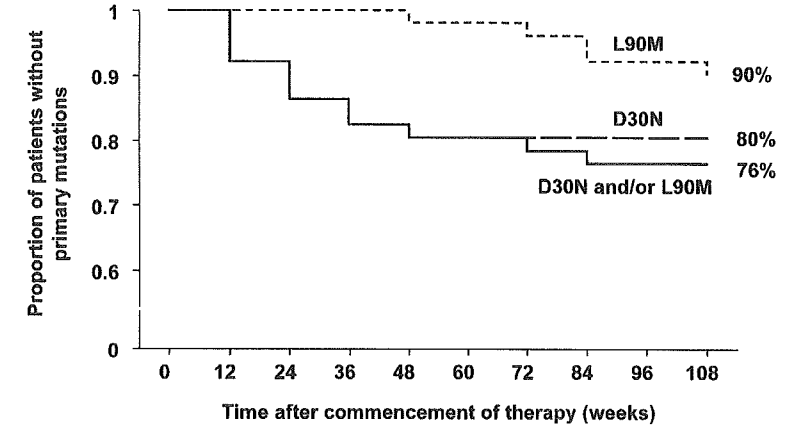
However, the rates of emergence of resistant mutations were not statistically different ($P = 0.188$). Table 3 shows the results of univariate Cox analysis for potential predictor of emergence of drug resistance. Although some factors had trends with approximately two-fold greater rate for drug resistance, none of the factors correlated with the emergence of NFV-associated resistance. This finding was probably due to the small number of patients who developed drug resistance.

4. Discussion

We described the rate of emergence of NFV-resistance virus in PI-naïve patients treated with NFV-containing regimens for up to 108 weeks in an HIV clinic cohort. In most clinical trials, the main outcome measure is viral suppression (virologic success) to undetectable level over a certain period of time (Staszewski et al., 1999; Grabar et al., 2000a; Gulick et al., 2000). However, such clinical trials did not refer to the emergence of drug resistance. Thus, this is the first study to investigate factors involved in drug resistance in daily clinical practice.

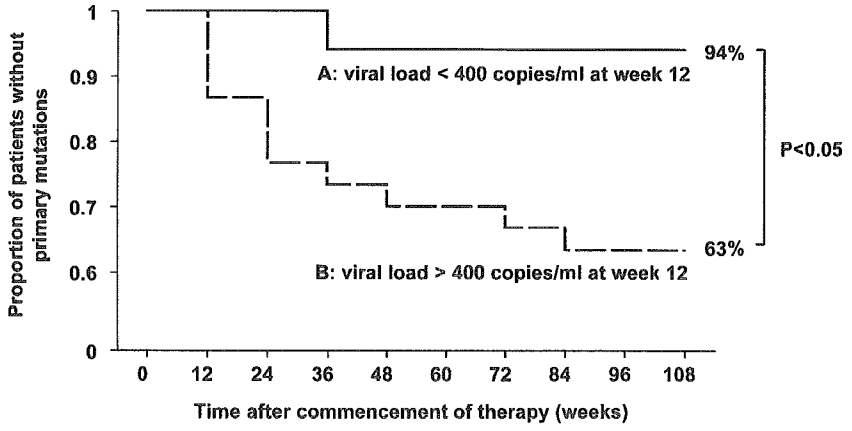
In the present study, rate of virologic success (viral load <400 copies/ml) was 63% at week 108 by ITT exposure. This rate is lower than the results of several clinical trials (Staszewski et al., 1999; Paredes et al., 2000) but quite similar to the results of unselected cohort studies (Fatkenheuer et al., 1997; Mocroft et al., 1998; Rhone et al., 1998; Paris et al., 1999; Grabar et al., 2000b). The most important predisposing factor for the virologic success is adherence to treatment (Lucas et al., 1999; Paris et al., 1999; Paterson et al., 2000). However, in daily clinical practice, patient population is heterogeneous in terms of clinical status,

such as CD4 count and viral load at baseline, prior AIDS diagnosis and prior treatment, and demographic status such as race, educational level and income, which might affect adherence to the



No. at risk										No. of cases with censored data	No. of cases with mutation
D30N	51	47	44	42	41	41	41	41	41	41	10
L90M	51	51	51	51	50	50	49	47	47	46	5
D30N and/or L90M	51	47	44	42	41	41	40	39	39	39	12

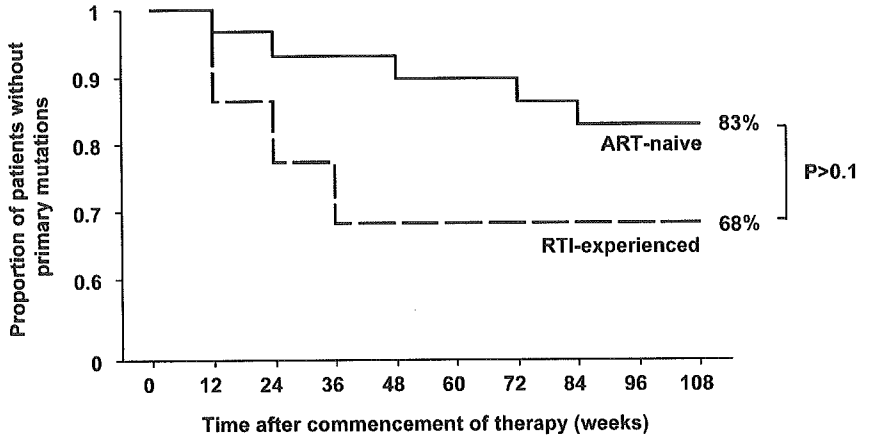
(A)



No. at risk											No. of cases with censored data	No. of cases with mutation
A	17	17	17	16	16	16	16	16	16	16	16	1
B*	30	26	23	22	21	21	20	19	19	19	19	11

(B)

Fig. 4. Kaplan–Meier estimates of the time to emergence of primary nelfinavir-associated resistance mutations. (A) Accumulation of D30N and/or L90M up to 108 weeks. Solid line: virus harboring D30N and/or L90M. Short line: D30N. Dash-line: L90M. (B) Patients were stratified into virologic success (viral load < 400 copies/ml) at week 12. Solid line: virologic success at week 12 ($n = 17$). Dash line: viral load remained > 400 copies/ml at week 12 ($n = 30$). *Four patients had discontinued their original regimens due to drug eruption by week 2. (C) Patients were stratified into antiretroviral therapy-naïve (ART-naïve) and reverse transcriptase inhibitor-experienced (RTI-experienced). Solid line: ART-naïve patients ($n = 29$). Dash line: RTI-experienced patients ($n = 22$).



No. at risk											No. of cases with censored data	No. of cases with mutation
ART-naive	29	28	27	27	26	26	25	24	24	24	24	5
RTI-experienced	22	19	17	15	15	15	15	15	15	15	15	7

(c)

Fig. 4 (*Continued*)

Table 3
Cox regression analysis for emergence of nelfinavir-associated resistance

	HR	95% CI	*P* value
Prior treatment			
ART-naïve	1.0	0.67–6.62	0.206
RTI-experience	2.10		
Clinical status			
Asymptomatic carrier	1.0	0.37–7.77	0.494
Prior AIDS diagnosis	1.70		
CD4 count at baseline			
> 200/μl	1.0	0.78–7.81	0.122
< 200/μl	2.48		
Viral load at baseline			
< 30,000 copies/ml	1.0	0.67–6.62	0.206
> 30,000 copies/ml	2.10		

HR, hazard ratio; 95% CI, 95% confidence interval; ART, anti-retroviral therapy; RTI, reverse transcriptase inhibitor.

treatment (Lucas et al., 1999). In our study, the clinical status was heterogeneous but demographic status was homogeneous. Therefore, adherence to treatment remained at high levels throughout the study in most patients, as determined by direct questioning.

Our study clearly demonstrated that prior RTI exposure, prior AIDS diagnosis, CD4 count < 200/μl at baseline and viral load over 30,000 copies/ml at baseline did not significantly influence the emergence of drug resistance. The stratified log-rank test revealed that virologic success at week 12 could predict treatment success over time without drug resistance. We have also previously noted the importance of viral load at week 12 with regard to the emergence of drug resistance (Aizawa et al., 1999). However, drug resistance assay for such patients becomes warranted in clinical practice when viral load exceeds 400 copies/ml at week 12. When these assays are not performed in such situations, viruses with multiple resistant mutations are likely to appear, indicating cross-resistance to other PIs (Tsuchiya et al., 2001). In this regard, a large clinical trial revealed that immunologic and virologic responses to 24-week HAART ensures a favorable clinical outcome (Grabar et al., 2000a). In our study, none of the patients with a viral load of <400 copies/ml at week 24 harbored NFV-resistant virus throughout the observation period. On the other hand, when viral load was >400 copies/ml at week 24, two-thirds of the resistant viruses had already appeared