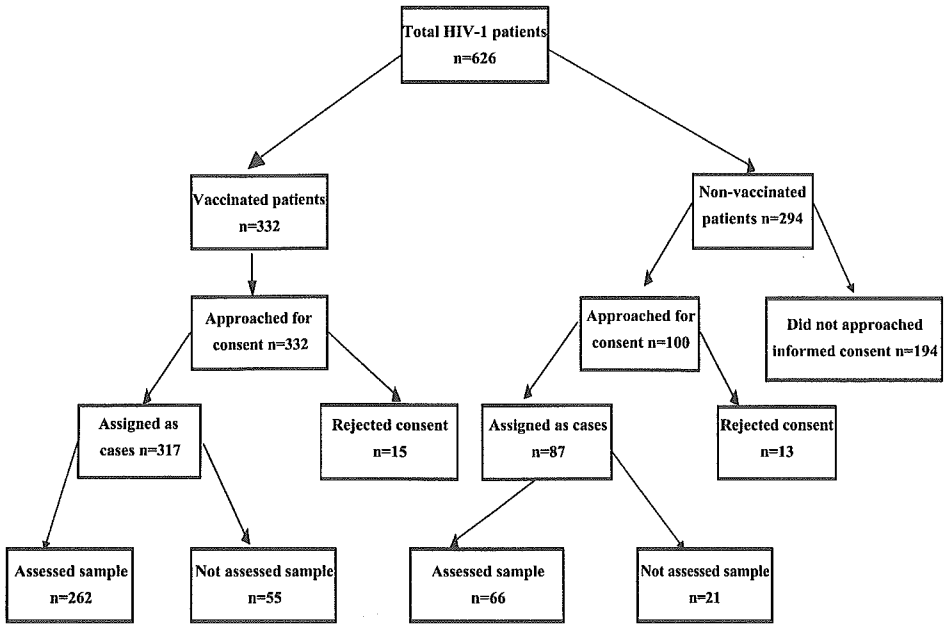

FIGURE 1. Profile of participants in this study.

4 with B strain); 3 by the symptoms, positive viral cultures, and antibody rise (2 with H1N1 strain and 1 with H3N2 strain); 5 by the symptoms, influenza test kit results, and antibody rise
(1 with H1N1 strain, 2 with H3N2 strain, and 2 with B strain); and 16 by the antibody rise between the symptoms (1 with H1N1 strain, 12 with H3N2 strain, and 3 with B strain). In total, 16 of 262 vaccinated patents had influenza illness (6.1%, confidence interval [CI]: 0.04–0.1) and 14 of 66 nonvaccinated patients had the illness (21.2%, CI: 0.13–0.35). The difference in the incidence between the 2 groups was significant ($P < 0.001$). The relative risk (RR) of influenza illness in vaccinated patients was 0.29 (CI: 0.14–0.55; $P < 0.001$) compared with nonvaccinated patients (Table 2). Eight patients who had a >4-fold rise in anti-H3 antibody titers between week 8 and week 16 without any clinical symptoms were not regarded as having influenza illness.

In patients with a CD4 count >200 cells/μL, the incidence of influenza illness in vaccinated patients (6.2%) was significantly lower than in nonvaccinated patients (21.0%) ($P < 0.001$). Conversely, in patients with a CD4 count <200 cells/μL, the same comparison showed no significant difference. Nevertheless, the incidences of influenza illness in vaccinated (5.9%) and nonvaccinated (22.2%) patients were the same as the incidence in patients with a CD4 count >200 cells/μL. Therefore, this analysis had lack of power because of the small number of nonvaccinated patients in this stratum. In vaccinated and nonvaccinated patients, the differences in the incidence were significant in patients with HAART ($P < 0.002$) and without HAART ($P < 0.05$) (see Table 2). When CD4 count was entered as a continuous variable, multivariate analysis using the logistic regression model identified vaccination ($P < 0.001$) and CD4 count ($P < 0.05$) but not HIV VL as independent predictors of influenza illness in HIV-1–infected patients.

In patients with influenza illness, 4 of 16 vaccinated patients and 4 of 14 nonvaccinated patients received an anti-influenza drug. None of the patients with influenza illness developed pneumonia that required treatment or hospitalization during the study period. Vaccination did not significantly change the HIV VL or CD4 count at weeks 8 and 16.

TABLE 1. Baseline Clinical and Immunologic Characteristics of Participants*

	Vaccinated	Nonvaccinated	*P*
No. participants (n)	262	66	—
Male/female ratio	7:1	15:1	n.s.
Median age, y (range)	41 (20–78)	40 (20–61)	n.s.
Received HAART (%)	75.2%	72.3%	n.s.
Median CD4 count at vaccination, μL (range)	380 (40–1137)	374 (66–1025)	n.s.
Median CD8 count at vaccination, μL (range)	778 (54–2649)	751 (163–1929)	n.s.
Median HIV VL at vaccination, $\log_{10}$/mL (range)	2.5 (1.5–6.2)	2.5 (1.5–6.4)	n.s.
Prior anti-H1 antibody-positive (%)	29.4%	26.4%	n.s.
Prior anti-H3 antibody-positive (%)	32.3%	30.3%	n.s.

*All participants were Japanese.
n.s. indicates not significant.

Anti-Hemagglutinin Antibody Responses Before and After Vaccination

HAI antibody titers against HA antigens (H1 and H3) were tested before and 8 and 16 weeks after vaccination (Table 3). To evaluate the effect of the single-shot influenza vaccine, subjects were divided into 2 groups based on the HAI titer before vaccination: the baseline HAI antibody-negative and antibody-positive groups. Furthermore, we excluded from this

TABLE 2. Incidence of Influenza Illness

	Vaccinated		Nonvaccinated		
	Illness/Patients	Rate (95% CI)	Illness/Patients	Rate (95% CI)	χ^2 Test
All patients	16/262	6.1% (0.04–0.1)	14/66	21.2% (0.13–0.35)	$P < 0.001$
CD4 count					
<200 cells/μL	3/51	5.9% (0.02–0.15)	2/9	22.2% (0.06–0.55)	n.s.
≥200 cells/μL	13/211	6.2% (0.03–0.1)	12/57	21.0% (0.12–0.33)	$P < 0.001$
HAART					
+	12/197	6.1% (0.04–0.1)	10/48	20.8% (0.11–0.34)	$P < 0.002$
−	4/65	6.2% (0.02–0.14)	4/18	22.2% (0.09–0.45)	$P < 0.05$

Incidence of influenza illness in healthy immunized controls was 3.8% (1 of 26, 95% CI: 0.01–0.19).
n.s. indicates not significant.

analysis the 13 patients who received the vaccination but had influenza illness (5 with H1N1 strain and 8 with H3N2 strain) during the study period so as to evaluate the antibody responses by the vaccination. The 8 patients who showed a >4-fold rise in anti-H3 antibody titers between week 8 and week 16 without any clinical symptoms were also excluded from this analysis, because the antibody rise in these cases was thought to be caused by influenza virus but not by vaccination. In the baseline HAI-negative group, the antibody responses to both antigens were significantly different compared with those in stratified HIV-1–infected patients by CD4 count (<200 cells/μL and ≥200 cells/μL; $P < 0.05$) at week 8 and week 16. These titers were low compared with those of the healthy immunized controls in both strata, however. In those with a CD4 count <200 cells/μL, 12 (27.9%) of 43 patients and 12 (32.4%) of 37 patients showed more than a 4-fold rise in the antibody responses against anti-H1 and anti-H3, respectively. In contrast, in those patients with a CD4 count >200 cells/μL, 62 (44.6%) of 139 patients and 61 (46.9%) of 130 patients showed a >4-fold rise in the antibody responses against anti-H1 and anti-H3, respectively. Although differences in the percentages of patients who showed both anti-H1 ($P = 0.05$) and anti-H3 ($P = 0.12$) antibody responses of the different CD4 strata were only marginal, there was a tendency for the single-shot vaccination to be more effective in terms of antibody responses in patients with a CD4 count >200 cells/μL. The antibody responses in both groups were not influenced by HIV VL (<100 copies/mL and ≥100 copies/mL; data not shown).

In the baseline HAI antibody-positive group, HAI titers to both antigens remained high and the sustainability of the antibody titers in HIV-1–infected patients was similar to those of the healthy controls, irrespective of CD4 counts (see Table 3). In terms of the antibody rise, in those with a CD4 count <200 cells/μL, 5 of 8 patients and 1 of 6 patients showed more than a 4-fold rise in the antibody response against anti-H1 and

TABLE 3. Anti-HA Antibody Responses After Vaccination in Baseline Anti-HA Antibody-Negative and Positive Individuals

	Anti-HA Antibody Responses* After Vaccination in HIV-1 Patients†						Healthy Immunized Controls	
	Stratum 1 (CD4 count <200 cells/μL)			Stratum 2 (CD4 count ≥200 cells/μL)				
	Week 0	Week 8	Week 16	Week 0	Week 8	Week 16	Week 0	Week 8
Baseline anti-H1 Ab-negative	n = 43			n = 139			n = 4	
Anti-H1 Ab responses	<10	26‡ (10–1280)	23‡ (10–1280)	<10	42 (10–1280)	36 (10–1280)	<10	135 (40–320)
Baseline anti-H3 Ab-negative	n = 37			n = 130			n = 4	
Anti-H3 Ab responses	<10	25‡ (10–640)	23‡ (10–1280)	<10	34 (10–1280)	32 (10–640)	<10	135 (40–320)
Baseline anti-H1 Ab-positive	n = 8			n = 67			n = 22	
Anti-H1 Ab responses	44 (20–320)	353 (40–1280)	208 (80–160)	54 (20–1280)	158 (20–1280)	143 (20–1280)	80 (20–640)	86 (20–640)
Baseline anti-H3 Ab-positive	n = 6			n = 73			n = 22	
Anti-H3 Ab responses	32 (20–80)	46 (20–160)	71 (20–640)	41 (20–1280)	105 (20–1280)	87 (10–1280)	59 (20–320)	66 (20–320)

*The data presented here are the geometric mean of anti-HA antibody titer. Range of the absolute titer is shown in parentheses.
†To analyze antibody responses to vaccination, patients with influenza infection were excluded from this analysis.
‡$P < 0.05$ compared with the respective value of stratum 2.
Ab indicates antibody. Change of the antibody titer from <10 to 40 U was considered a 4-fold rise.

anti-H3. Conversely, in those with a CD4 count >200 cells/μL, 16 of 67 patients and 19 of 73 patients showed more than a 4-fold rise.

Anti-H1 and Anti-H3 Antibody Responses in Patients With Influenza Illness Despite Vaccination

A total of 16 patients (5 with H1N1 strain, 8 with H3N2 strain, and 3 with B strain) had influenza illness among the vaccinated group during this study period. In the 5 patients with H1N1 illness, 3 were baseline anti-H1 antibody-negative and 2 had the antibody. Among the 3 baseline anti-H1 antibody-negative patients, 2 were infected before week 8 and 1 was infected after week 8. In the patient infected after week 8, no anti-H1 antibody was detected at week 8. In each of the 2 baseline anti-H1 antibody-positive patients, the titer was 20 U. Both patients were infected before week 8. In the 8 patients with H3N2 illness, 6 were baseline anti-H3 antibody-negative and 2 were positive for the antibody. In the 6 baseline anti-H3 antibody-negative patients, all were infected after week 8. Among these 6 patients, 4 were negative for anti-H3 antibody at week 8, whereas 2 had a 4-fold rise in the antibody before infection. In each of the 2 baseline anti-H3 antibody-positive patients, the titer was 20 U. Both patients were infected after week 8. Anti-H3 antibody at week 8 was increased to 40 U (a 2-fold rise) only in 1 patient. Overall, among the 9 infected patients (1 with H1N2 strain and 8 with H3N2 strain) in whom the antibody responses at week 8 could be evaluated, only 2 had a >4-fold rise of the antibody response before infection.

H1-Specific CD4 T-Cell Response Before and After Vaccination in Baseline Anti-H1 Antibody-Negative Subjects

H1-specific CD4 T-cell responses at week 8 were HIV VL dependent ($P < 0.005$) but not CD4 count dependent (Fig. 2A). Therefore, H1-specific CD4 T-cell responses were significantly increased by vaccination in HAART-treated patients ($P = 0.001$), because HIV VL was decreased by HAART (see Fig. 2B). In contrast, responses of HAI antibody titer were not different between HAART-treated and antiretroviral-naive patients (see Fig. 2C).

Comparison of Immune Responses to H1 Antigen at Week 8 Between Influenza A/H1N1–Infected and –Uninfected Patients

Five individuals were infected with influenza A/H1N1 during this season. HAI antibody titers at 8 weeks after the vaccination were not different between the infected and uninfected individuals. In contrast, H1-specific CD4 T-cell responses at week 8 were significantly low in the infected persons compared with those in the uninfected persons ($P < 0.05$; Fig. 3).

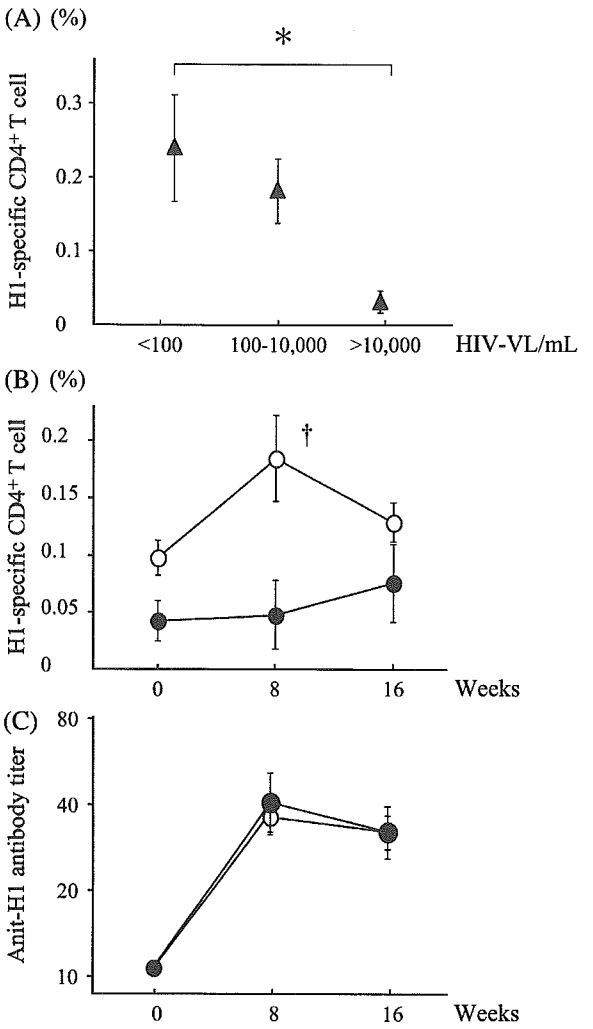

FIGURE 2. H1-specific CD4+ T-cell responses after influenza vaccine in baseline anti-H1 antibody–negative patients. A, Correlation of plasma HIV-1 viral load (HIV VL) and percentage of H1-specific CD4+ T cells. *H1-specific CD4+ T cells (▲) were significantly fewer in number in subjects with an HIV VL >10,000 copies/mL ($P < 0.005$). The number of samples with an HIV VL <100 copies/mL was 53, there were 19 samples with 100 to 10,000 copies/mL, and there were 11 samples with >10,000 copies/mL, because H1-specific CD4+ T cells were only examined in the first 10 samples per day as stated in the text. B, Changes in the percentage of H1-specific CD4+ Tcells in highly active antiretroviral therapy (HAART)–treated; (○; n = 63) and antiretroviral-naive patients (●; n = 12). †HAART-treated patients had significantly greater numbers of H1-specific CD4+ T cells at week 8 ($P < 0.01$) than antiretroviral-naive patients. C, Changes in anti-H1 antibody titer in HAART-treated (○; n = 131) and antiretroviral-naive patients (●; n = 35). Anti-H1 antibody responses were similar in both groups. Data are mean ± SEM.

DISCUSSION

Our prospective study confirmed many conclusions of previously reported small studies. First, we confirmed the protective effect of influenza vaccine in HIV-1–infected patients.[8–15] Second, anti-H1–specific and anti-H3–specific antibody responses were examined in HIV-1–infected patients after vaccination, and the responses were confirmed to be dependent on CD4 counts.[8–11]

To clarify the efficacy of a single-shot vaccination, we divided the participants by the positivity of anti-H1– and anti-H3–specific antibodies before vaccination and found that in

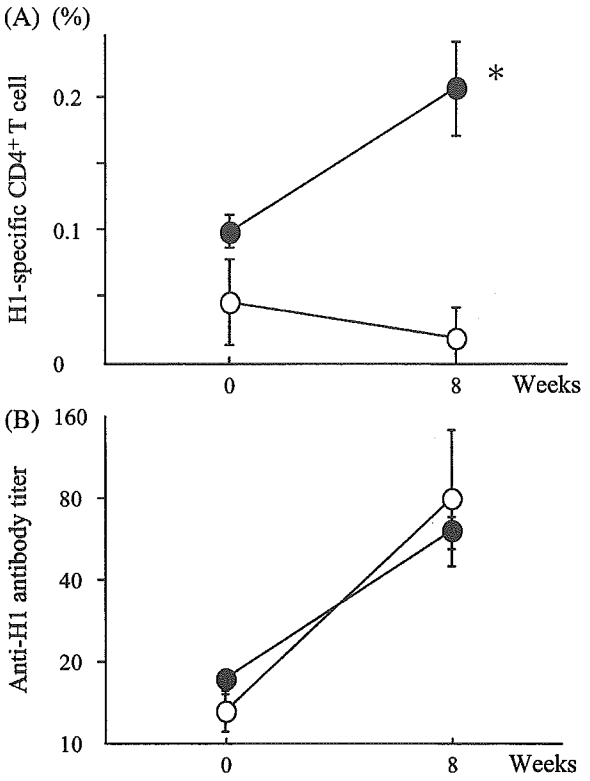

FIGURE 3. H1-specific CD4+ T cells and anti-H1 antibody responses at week 8 after vaccination in influenza A/H1N1–infected patients. Five vaccinated individuals were infected with influenza A/H1N1. A, Percentage of H1-specific CD4+ T-cell responses in infected (○; n = 4) and noninfected (●; n = 119) individuals. *H1-specific CD4+ T cells responded better to influenza A/H1N1 in noninfected patients than in infected patients ($P < 0.05$). One sample of 5 influenza A/H1N1–infected individuals was not examined because the sample was not among the first 10 samples per day as stated in the text. B, Anti-H1 antibody titers in infected (○; n = 5) and noninfected (●; n = 249) individuals. The anti-H1 antibody response at week 8 was similar in both groups. Data are mean ± SEM.

baseline antibody-negative HIV-1–infected patients, the antibody responses to the single-shot vaccination were less effective than those in healthy patients. In contrast, however, in baseline antibody-positive HIV-1–infected patients, the antibody responses were similar or more effective than those in the healthy controls and the titers exceeded >40 U in most cases, irrespective of CD4 count. Previous studies demonstrated that an antibody titer >40 U could be used as an index of vaccine protection.[12,25] In our study, the antibody titer was <40 U in most patients who became infected with influenza. Considered together, these results suggest that the antibody response may support the clinical efficacy of influenza vaccination. Kroon et al[8] reported that postvaccination antibody titers were higher in previously vaccinated HIV-1–infected patients than in nonvaccinated patients, although the difference was not significant. In the present study, the antibody titers showed a better response in individuals positive at baseline for anti-HA antibody than in those negative for the antibody. Furthermore, the response was well sustained, irrespective of CD4 count. Thus, it is conceivable that annual vaccination is specifically important for all HIV-1–infected patients. Sustainability of the antibody titer raised by the vaccination is to be followed in a future study.

In the immunologic part of our study, we examined antibody responses and specific CD4 T cells. The antibody response was almost the same as that reported previously[8,9]; the response correlated with the CD4 count. In contrast, specific CD4 T cells were much more influenced by HIV VL than by CD4 count.[1,8–15] Therefore, the specific CD4 T cells were higher in patients treated with HAART than in those untreated. This result indicates that HAART improves HA-specific CD4 T cells like in other infections,[21] or, in other words, the heightened cellular response to the influenza vaccine suggests functional reconstitution of the immune system after HAART.

Our data indicate that the specific CD4 T-cell responses may be related to HIV VL. The specific CD4 T-cell response needs antigen presentation by dendritic cells.[26] HIV-1 infection impairs the function of antigen presentation of dendritic cells.[27] Therefore, specific CD4 T-cell responses may be profoundly decreased in patients with a high HIV VL.

It is interesting to note that the percentage of H1-specific CD4 T cells at week 8 was significantly lower in influenza A/H1N1–infected patients. It is conceivable that the response of HA-specific CD4 T cells at week 8 can predict the efficacy of influenza vaccine. Influenza-specific CD4 T cells provide help (as Th cells) to B cells for the production of antibody to influenza HA and neuraminidase[28,29] and also promote the generation of virus-specific CD8+ cytotoxic T lymphocytes (CTLs).[26,30–33] Therefore, the specific CD4 T cell must have a protective role. This concept would be more reliable if we had analyzed H3-specific CD4 T cells rather than H1-specific CD4 T cells, because influenza A/H3N2 was the predominant subtype in this season. Further studies are necessary to elucidate this point.

Our study was designed as a prospective but nonrandomized study, because influenza vaccine has been already recommended for HIV-1–infected patients.[7] Practically, the number of nonvaccinated patients who did not participate in our study was higher than that of vaccinated patients (13% of nonvaccinated patients vs. 4.5% of vaccinated patients), and the violation rate of the study protocol was higher in nonvaccinated patients than in vaccinated patients (24.1% vs. 17.4%). Thus, 262 (78.9%) of 332 vaccinated patients and 66 (66%) of 100 nonvaccinated patients were analyzed in this study. Although a relatively high proportion of patients failed to complete the protocol, the main reason for the drop out may have been the lack of incentives and the need to visit our clinic on a fixed date for blood sampling. The vaccinated and nonvaccinated groups were well balanced in terms of baseline characteristics, however. Finally, we believe that the selection bias of participants, if any, is negligible.

In conclusion, our prospective study in a large population demonstrated that influenza vaccine provides protection of HIV-1–infected patients. In baseline antibody-negative patients, the antibody responses to the vaccination were significant in those patients with a CD4 count >200 cells/μL compared with those with a CD4 count <200 cells/μL. In contrast, in baseline antibody-positive patients, good antibody responses were observed, irrespective of CD4 counts. Annual vaccination of

HIV-1–infected patients is thus recommended. Therapy with HAART improves the cellular immune response to influenza HA.

ACKNOWLEDGMENTS

The authors are indebted to Dr. N. Sugaya from the Keiyu Hospital for helpful suggestions regarding this study and to Drs. T. Gotanda and Y. Suzuki from the Kitasato Institute Research Center for Biologicals for kindly providing purified HA obtained from influenza virus A/New Caledonia/20/99. The authors thank Dr. N. Ishizuka, International Medical Center of Japan, for special advice on interpretation of the statistical analyses. We also thank F. Negishi and Y. Takahashi for sample stock, preparation, and technical support.

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APPENDIX

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発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Tsuchiya K, Matsuoka-Aizawa S, Yasuoka A, Kikuchi Y, **Tachikawa N**, Genka I, Teruya K, Kimura S, and Oka S.	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.	***J Clin Virol***	27	252-262	2003

ELSEVIER

Journal of Clinical Virology 27 (2003) 252–262

JOURNAL OF CLINICAL VIROLOGY

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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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Received 15 February 2002; accepted 20 August 2002

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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1386-6532/02/$ - see front matter
PII: S1386-6532(02)00179-8

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′-CCAACAGCCCCAC-CAGA-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

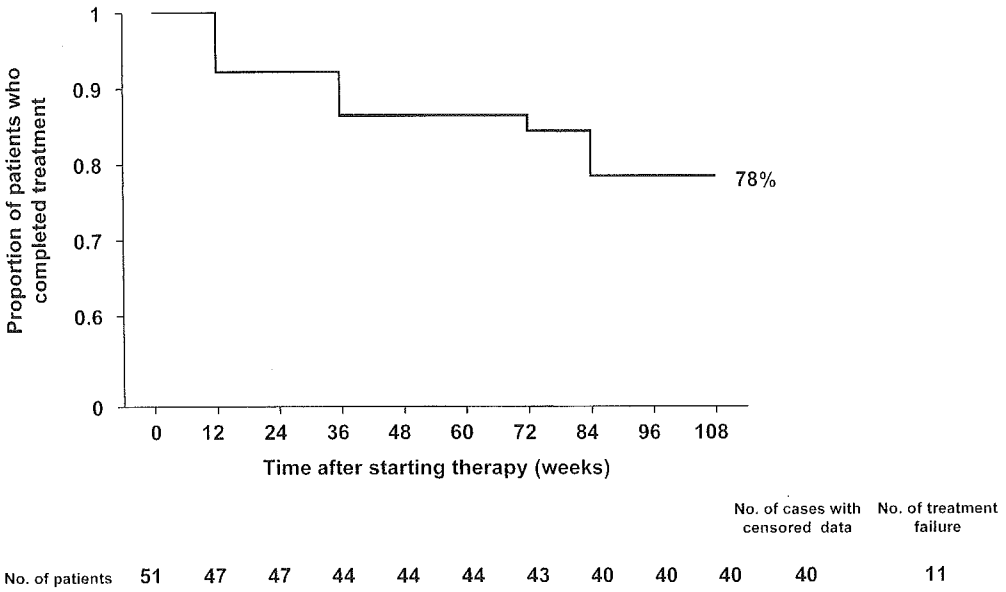


Fig. 1. Kaplan–Meier estimates of the time to discontinuation of nelfinavir-containing regimens.

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.

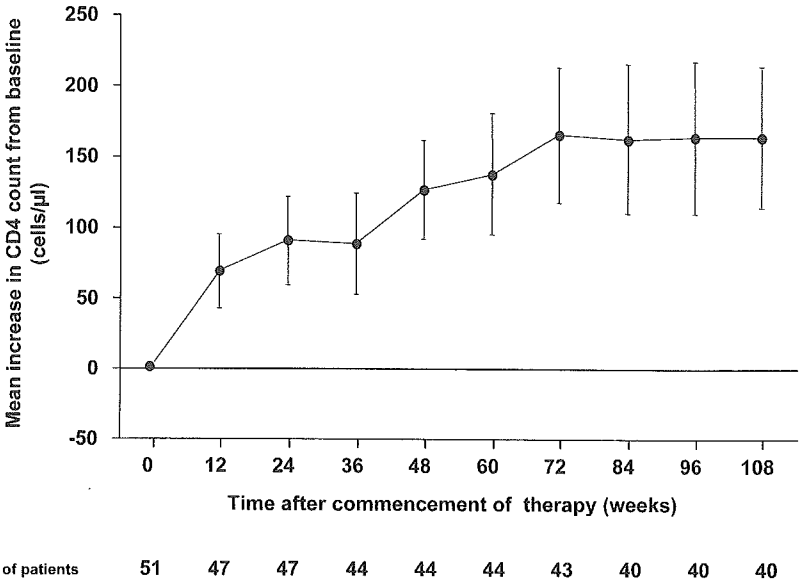


Fig. 2. Mean changes in CD4 count from baseline. Vertical bars represent the 95% confidence interval.

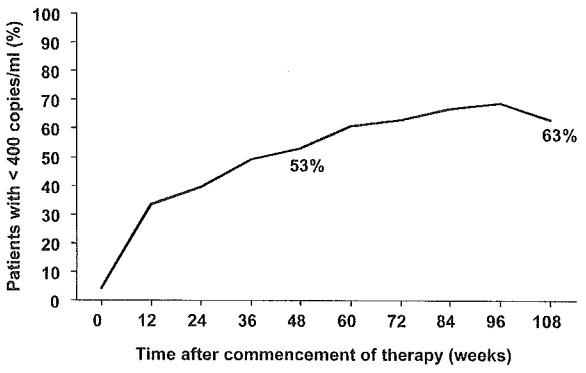


Fig. 3. Percentage of patients with plasma viral load <400 copies/ml.

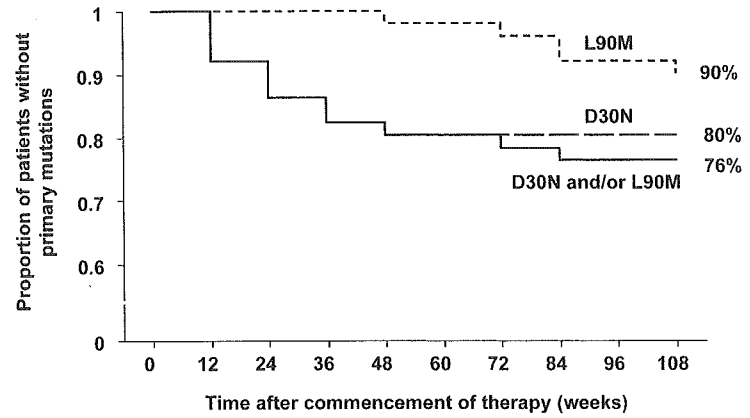
However, the rates of emergence of resistant mutations were not statistically different ($P = 0.188$). Table 3 shows the results of univariate Cox analysis for potential predictor of emergence of drug resistance. Although some factors had trends with approximately two-fold greater rate for drug resistance, 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.

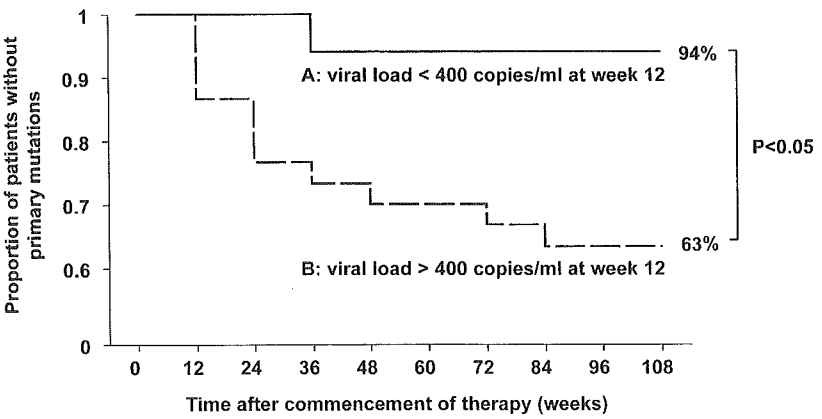
In the present study, rate of virologic success (viral load <400 copies/ml) was 63% at week 108 by ITT exposure. This rate is lower than the results of several clinical trials (Staszewski et al., 1999; Paredes et al., 2000) but quite similar to the results of unselected cohort studies (Fatkenheuer et 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

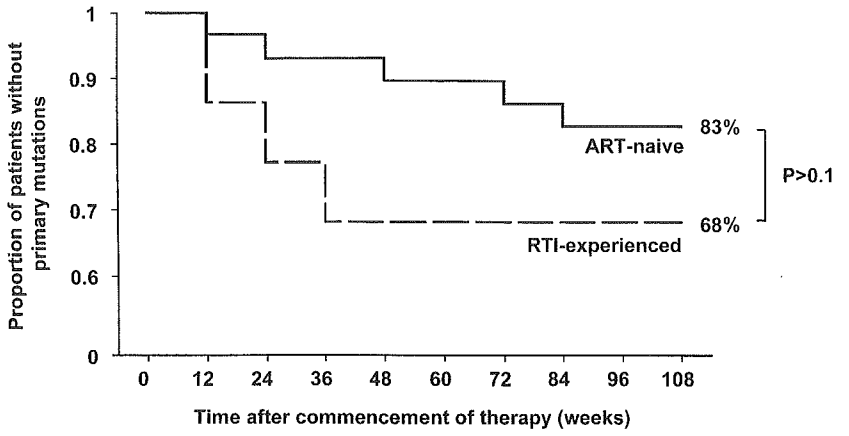
(A)



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

at that stage. Therefore, to avoid the emergence of drug resistance, there is a need for close monitoring of viral load and possible change of the treatment regimen before week 24 of NFV-containing therapy if viral load remains >400 copies/ml at week 12. In the present study, we did not address the influence of combination therapy on drug resistance because of the small number of patients. In this cohort, AZT plus 3TC or d4T plus 3TC were the main drugs used in combination with NFV (Table 1). In this regard, Squires et al. (2000) demonstrated that these two combinations do not affect the clinical efficacy.

NFV was well tolerated during the clinical trial. Only 4% of 696 patients discontinued the treatment by 24 weeks as a result of adverse events (Markowitz et al., 1998). In contrast, 10% ($n = 5$) of our patients discontinued treatment due to adverse events related to NFV. Among them, four stopped NFV due to drug eruptions, which appeared within 2 weeks of treatment. In the absence of such reaction soon after commencement of therapy, adherence to treatment with NFV-containing regimens was noted in such patients. Six patients changed their treatment due to virologic failure. However, new therapeutic regimens resulted in the suppression of viral load to undetectable in all cases according to their resistant profiles, indicating the importance of drug resistance testing in clinical practice (Gatanaga et al., 1999; Hirsch et al., 2000).

In conclusion, our results showed that NFV-containing regimens for PI-naïve patients are safe and effective when taken up to 108 weeks in daily clinical practice. There was no independent predictor for the emergence of drug resistance in clinical status, such as CD4 and viral load at baseline, prior AIDS diagnosis and prior use of RTI. Therefore, NFV can be used widely in PI-naïve patients. Virologic success at week 12 could predict continuation of treatment without the appearance of primary mutations thereafter.

Acknowledgements

The authors thank Y. Takahashi for serum stock. We also thank the clinical and laboratory staffs, and especially the coordinator nurses of the AIDS Clinical Center, International Medical Center of Japan, for their helpful support. This work was supported in part by a grant-in-aid for AIDS research from the Ministry of Health, Labour and Welfare of Japan (H12-AIDS-001) and the Organization of Pharmaceutical Safety and Research (96-1).

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Functionally Impaired HIV-Specific CD8 T Cells Show High Affinity TCR-Ligand Interactions[1]

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We eventually isolated two different clonotypic CD8 T cell subsets recognizing an HIV Pol-derived epitope peptide (IPLTEEAEL) in association with HLA-B35 from a chronic HIV-infected patient. By kinetic analysis experiments, the subsets showed a >3-fold difference in half-lives for the HLA tetramer in complex with the Pol peptide. In functional assays in vitro and ex vivo, both subsets showed substantial functional avidity toward peptide-loaded cells. However, the high affinity subset did not show cytolytic activity, cytokine production, or proliferation activity toward HIV-infected cells, whereas the moderate affinity one showed potent activities. Furthermore, using ectopic expression of each of the TCR genes into primary human CD8 T cells, the CD8 T cells transduced with the high affinity TCR showed greater binding activity toward the tetramer and impaired cytotoxic activity toward HIV-infected cells, corroborating the results obtained with parental CD8 T cells. Taken together, these data indicate that impaired responsiveness of T cells toward HIV-infected cells can occur at the level of TCR-ligand interactions, providing us further insight into the immune evasion mechanisms by HIV. *The Journal of Immunology,* **2004, 173: 5451–5457.**

Our understanding of how HIV avoids control by the human immune system remains incomplete. Although $CD8^+$ CTL are believed to have an important role in the immunopathogenesis of HIV-1 infection, it is not completely clear why viral replication persists and progressive immunodeficiency generally ensues (see recent reviews, 1–5). The findings of several studies show that HIV-specific CTL taken ex vivo can have functional defects that could undermine their control of the virus. For example, whereas most HIV-specific CD8 T cells in patients with chronic HIV disease produced antiviral cytokines on contact with cognate Ag, these cells showed diminished perforin expression and capacity for proliferation compared with CMV-specific T cells (6) and T cells in long term nonprogressors (7), respectively. Such different functional outcomes in T cells can be caused by the quality of T cell activation, such as the strength of TCR engagement and costimulatory or inhibitory interactions (8–10). The kinetics and affinity of interaction between TCR and peptide-MHC complex (pMHC)[3] are the basis of T cell activation. For the most part, longer half-lives of TCR-pMHC interaction correspond to higher T cell activation (11–14). However, in the case of some peptide variants as well as mutations in MHC and/or TCR, a longer half-life was reported to weaken T cell reactivity (15–18). Moreover, it remains unclear what are the functional roles of peripheral T cell subsets that bear TCR with high affinity for a MHC ligand in association with a foreign peptide, because T cells with high affinity for a foreign pMHC appear to be negatively selected in the thymus and not exported to the periphery (19).

In contrast, we and others have generated many CTL lines and clones from HIV-infected patients that were cytotoxic toward HIV-infected cells in vitro in the course of experiments to identify HIV-derived CTL epitopes in previous studies (20). Given that only T cells that were positive for epitope-specific cytolytic activity were reported in these studies, we hypothesized that T cells with negative cytolytic activity toward HIV-infected cells, which may reflect the loss of antiviral effector functions of HIV-specific CTLs in vivo, were concurrently generated, but not further examined, due to their negative activity. Therefore, to examine cell-based mechanisms involved with impaired functions of HIV-specific CD8 T cells, we have again been testing CD8 T cell clones isolated from HIV-infected patients for their lack of killing activity toward HIV-infected cells even though they retained their specificity toward HIV Ags.

In the present study we focused on remarkable functional differences in two different CD8 $\alpha\beta$ T cell subsets (TCR $V\alpha12^+$ and $V\delta1^+$) specific for an HIV Pol-derived epitope peptide (IPLTEEAEL) from a chronic HIV-infected patient. Interestingly, the subsets showed a >3-fold difference in binding activity toward the HLA tetramer in complex with the Pol peptide. The high affinity subset ($V\delta1^+$) showed impaired reactivity toward HIV-infected cells in vitro and ex vivo, whereas the moderate affinity subset ($V\alpha12^+$) had potent reactivity. Additional genetic transfer of each of these TCR genes into human primary CD8 T cells clearly indicated that impaired responsiveness of T cells toward HIV-infected cells can occur at the level of TCR-ligand interactions.

Materials and Methods

Tetramer binding assay

The CTL lines (5×10^4 cells; >60% $tetramer^+$ $CD8^+$ cells) generated by repeated stimulation of the patient's lymphocytes (*HLA-A**2402/*A**2601, *HLA-B**3501/*B**5101) with the Pol peptide (IPLTEEAEL) were first stained with various concentrations of the tetramer at 4 or 37°C for 15 min. The cells were subsequently stained at 4°C for 15 min with anti-CD8-PerCP (BD Pharmingen, San Diego, CA), FITC-conjugated anti-Vα12

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Received for publication May 6, 2004. Accepted for publication August 12, 2004.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by grant-in-aids for scientific research from the Ministry of Education, Science, Sports, and Culture of Japan and by grant-in aids for AIDS research from the Ministry of Health, Labor, and Welfare of Japan. This research was also supported in part by a grant from the Japan Health Science Foundation.

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[3] Abbreviations used in this paper: pMHC, peptide-MHC complex; IRES, internal ribosome entry site; MFI, mean fluorescence intensity.

0022-1767/04/$02.00

TCR (Serotec, Oxford, U.K.), and anti-Vδ1 TCR mAbs. The anti-Vδ1 TCR mAb (A13; provided by L. Moretta, Istituto di Istologia ed Embriologia Generale, Genova, Italy) (21) had been labeled with PE-conjugated Fab specific for the Fc portion of mouse IgG1 (Molecular Probes, Eugene, OR). For the kinetic analysis of tetramer binding, the CTL line was first incubated with 5 μM tetramer at 4°C. A portion of the reaction was removed periodically (2, 5, 10, 15, 30, and 60 min), and the cells were subsequently stained with anti-CD8 and anti-TCR mAbs as described above. For kinetic analysis of the tetramer dissociation, a CTL line was stained with 5 μM tetramer for 60 min at 4°C. Then cells were rapidly washed twice and suspended in 1.5 ml of a buffer (2% BSA in PBS) supplemented with a blocking Ab. A portion of the reaction was then removed periodically (2, 5, 10, 15, 30, and 60 min), and the cells were subsequently stained with the anti-CD8 and anti-TCR Abs. For the flow cytometric analysis, $V\alpha12^+$ or $V\delta1^+$ $CD8^+$ cells were gated and then assessed for their tetramer binding level.

Cytotoxic assay

The cytotoxic activity of CTL clones generated previously (22, 23) was determined by a standard ^{51}Cr release assay as previously described (22). For Pol peptide-pulsed target cells, ^{51}Cr-labeled C1R-B*3501 cells were pulsed with the peptide for 1 h, then incubated with the effector T cells for an additional 4 h at 37°C. For virus-infected target cells, C1R-B*3501 cells or .221-B*3501 cells expressing human CD4 Ag were infected with HIV-1 GagPol-expressing vaccinia virus, HIV-1 LAI, or vesicular stomatitis virus envelope glycoprotein-pseudotyped HIV-1 HXB2D. Note that all these viruses have the same epitope sequence as that used for synthetic Pol peptide (IPLTEEAEL). The cells were subsequently labeled with ^{51}Cr and incubated with the effector T cells for 6 h at 37°C. It should be noted that >70% cells expressed the p24 Gag Ag, as revealed by intracellular flow cytometric analysis of target cells.

Cytokine secretion assay

CTL clones were cocultured with .221-CD4-B*3501 cells, either pulsed with various concentrations of the Pol peptide or infected with HIV-1 LAI for 2 h at 37°C. Brefeldin A (10 μg/ml) was then added, and the culture was continued for an additional 4 h. Then the cells were permeabilized, stained with anti-IFN-γ and TNF-α mAbs (BD Pharmingen), and analyzed by flow cytometry as previously described (23).

Ex vivo activation assay

Cryopreserved PBMC of HIV-positive (1×10^6) or negative donors (5×10^6) were stained with the tetramer at 37°C for 15 min, followed by anti-CD8 and anti-TCR Abs at 4°C for 15 min. The cryopreserved PBMC of the HIV-positive patient were stimulated, or not, with irradiated .221-CD4-B*3501 cells, either pulsed with 100 nM Pol peptide or infected with HIV-1 LAI (>70% p24 Gag^+). The cells were cultured at 37°C for 12 days in RPMI 1640 supplemented with 10% FCS and 200 U of IL-2. A portion of the stimulated cells (2×10^5) was stained as described above.

Construction of retroviral vectors and gene transfer

The genes encoding full-length α and β TCR of CTL 55 (23) and 589 (22) were subcloned into the pGC-based retroviral vector (pGCDNsap[MSCV]; provided by M. Onodera, Tsukuba University, Ibaragi, Japan) (24). The sequence data of the TCR genes are available from DDBJ under accession numbers AB164056, AB164057, AB164620, and AB164621. The genes encoding a murine heat-stable Ag (CD24) or a GFP were also incorporated into the constructs with an internal ribosome entry site (IRES) following the α or β TCR gene to facilitate monitoring of the expression of the α or β TCR gene, respectively, in the transduced cells.

Human primary CD8 T cells were isolated from PBMC of an HIV-negative healthy donor with *HLA-B*3501* using anti-CD8 magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). The resultant CD8 T cells were activated over a 3-day period by anti-CD3 mAb (OKT3) coated on the culture dish, then transferred to recombinant fibronectin-coated plates (Takara Shuzo, Otsu, Japan) and incubated for 72 h with the retrovirus supernatant containing the TCR α-IRES-CD24 gene. Transduced T cells expressing CD24 Ags were isolated using PE-labeled anti-CD24 mAb (BD Pharmingen) and anti-PE magnetic beads (Miltenyi Biotec). The isolated cells (>80% of the cells were $CD8^+$ $CD24^+$) were subsequently transduced with another construct containing TCR β-IRES-GFP as described above.

Results

Functional difference in CTL clones in response to HIV-infected cells in vitro

An HIV Pol peptide (IPLTEEAEL) is a CTL epitope endogenously presented by HLA-B*3501 (20). In Pol peptide-stimulated lymphocytes from a patient with chronic HIV infection, we generated two $CD8^+$ αβ T cell clones, designated CTL 55 and 589, that were shown to express TCR Vδ1.1/Vβ13.3 and Vα12.1/Vβ5.6, respectively, on their cell surface (22, 23). It is of note that the genes encoding a Vδ1 variable segment are expressed in ~0.5% of peripheral $CD8^+$ αβ T cells in human healthy individuals and that the Vδ1-bearing TCR αβ recognizes a peptide presented by HLA class I molecules (21, 23). The functional properties of both T cell clones were first tested for their cytotoxic and cytokine production activities in response to cells either pulsed with the Pol peptide or infected with viruses expressing HIV Pol proteins. Peptide titration experiments showed that both clones had substantial cytotoxic activities (Fig. 1*A*). It should be noted that in repeated experiments, CTL 589 appeared to

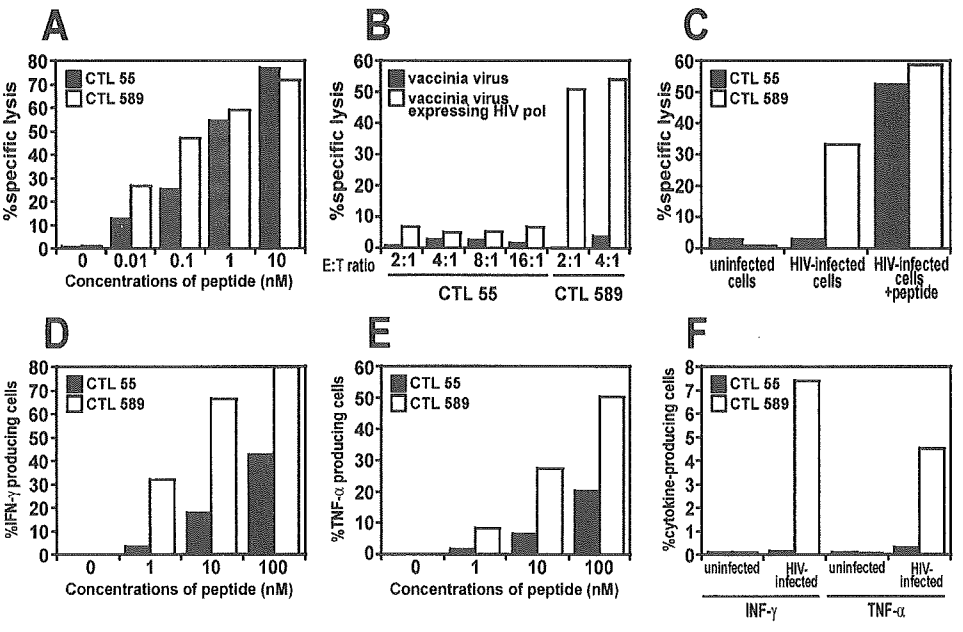

FIGURE 1. Analysis of effector functions of the CTL clones. *A* and *B*, Cytotoxic activity of CTL 55 and 589 toward C1R-B*3501 cells either pulsed with the indicated concentrations of the Pol peptide (*A*) or infected with vaccinia virus expressing HIV-1 GagPol polyproteins at the indicated E:T cell ratios (*B*). *C*, Cytotoxic activity of CTL 55 and 589 toward .221-B*3501 cells uninfected or infected with HIV-1 in the absence or the presence of 100 nM Pol peptide at an E:T cell ratio of 2:1. *D–F*, Intracellular staining for IFN-γ and TNF-α of CTL 55 and 589 in response to .221-B3501 cells either pulsed with the indicated concentrations of the Pol peptide (*D* and *E*, respectively) or infected with HIV-1 at an E:T cell ratio of 1:1 (*F*). Data are shown as the means of duplicate assays for at least three independent experiments.