HLA-A*2402 and that Nef138-10-specific CTLs failed to kill target cells infected with HIV-1 recombinant Sendai virus containing the 2F mutant; data suggested that the 2F is a mutation for escape from the specific CTLs (20). However, the question remained as to whether Nef138-10-specific CTLs can mediate strong immune pressure on HIV-1 replication so that they select the 2F mutant in vivo. In the present study, we clarified this question by investigating the abilities of Nef138-10-specific CTL clones to suppress replication of the WT and the 2F mutant viruses. Each Nef138-10-specific CTL clone exhibited a strong ability to suppress replication of Nef$^+$ HIV-1 at E-to-T ratios of 1:1 and 0.1:1. This ability is much stronger than that of most HIV-1-specific CTLs (19, 37, 38), suggesting that these CTLs can mediate strong immune pressure in vivo. In addition, our study using the HIV-1 2F mutant showed that Nef138-10-specific CTLs failed to kill target cells infected with the 2F mutant and to suppress replication of the mutant, confirming that 2F is an escape mutant.

Escape mutations occur at sites within CTL epitopes, where the substitution of an amino acid abrogates HLA binding, reduces the recognition of the TCR, and/or interferes with efficient Ag processing (14, 41). The 2F mutant peptide bound to HLA-A*2402 molecules with an efficiency similar to that of the WT peptide. Both the WT and the 2F tetramers bound to Nef138-10-specific CTL clones. In addition, Nef138-10-specific CTL clones killed target cells prepulsed with Nef138-10-2F peptide. These findings suggest that the escape mechanism of the 2F mutant involves the disruption of cellular processing of the 2F mutant peptide. However, since Nef138-10-2F-specific CTL clones effectively killed the target cells infected with the 2F mutant virus and suppressed the replication of the mutant virus, the 2F peptide can be naturally processed and presented by HLA-A*2402. The 2F-specific CTL clones could recognize the 2F peptide much more effectively than the WT peptide, whereas the clones showed much stronger abilities to suppress replication of the WT virus than that of the 2F mutant virus. These observations indicate that the change from Tyr to Phe remarkably reduced the presentation of the epitope peptides in Ag processing but that the 2F mutant could still be presented in the cells infected with the mutant.

It is well known that in both HIV-1 and SIV infections, escape mutations are poorly recognized in new hosts who share the same HLA alleles with donors (17, 32). If escape mutant peptides fail to bind to HLA class I restriction molecules or the mutation critically affects the Ag processing, these escape mutants are hardly recognized and fail to elicit the specific CTLs in new hosts sharing the same HLA alleles. On the other hand, if escape mutant peptides can bind to HLA class I restriction molecules and can be processed and presented, it remains possible that the mutant epitope is recognized in new hosts. The 2F mutant peptide effectively bound to HLA-A*2402 (Fig. 3B), suggesting the possibility that the 2F mutant peptide is presented by HLA-A*2402. We therefore selected the three donors who were infected with the 2F mutant virus at an early phase (within 10 weeks before the first visit) and investigated whether the 2F-specific CD8$^+$ T cells were elicited in these donors. It was strongly suggested that these patients had been infected with the 2F virus, since in the donors who had been infected with the WT virus, the 2F mutation was selected approximately 2 years after infection. The 2F-specific CD8$^+$ T cells were elicited in these three donors, although this mutant epitope was very weakly presented by HLA-A*2402. Thus, escape mutant-specific CTLs can be elicited in new hosts even if the mutant epitope peptide is very weakly presented.

The reversion of a CTL escape mutation to the WT occurs when the mutant virus is transmitted to a new host not sharing HLA class I alleles (18, 29) and even to a new host sharing HLA class I alleles with the monkey donors before the specific CTL is elicited (8, 27). Although the reversion of the 2F epitope to the WT one was reported for chronically HIV-1-infected individuals having no HLA-A*2402, the rate of reversion was very low (20), suggesting that the Y-to-F substitution does not inflict a large fitness cost on HIV-1. A previous study showed that the 2F mutant was still detectable in 56% of HLA-A*2402$^-$ Japanese patients infected through USI (20). In contrast, in the present study, it was found in only 31% of the patients. This difference between these two studies may have resulted from the difference in the time when the sequence was analyzed after the infection. Although the frequency of the 2F mutant in HLA-A*2402$^-$ Japanese individuals is different between the two studies, the studies indicate that 2F mutant did accumulate in HLA-A*2402$^-$ Japanese individuals infected through USI. The reversion of this epitope should occur but may be very slow in HLA-A*2402$^-$ donors. It is thought that the reversion does not occur in HLA-A*2402$^+$ individuals, because the 2F-specific CTLs can strongly suppress replication of the WT virus. Indeed, the reversion was not found in three patients who had been primarily infected with the 2F mutant virus and monitored for 2 to 3 years. Thus, the 2F mutant is accumulating in the Japanese population, of which 70% carry HLA-A*2402.

The competitive tetramer binding assay using the two tetramers could distinguish CD8$^+$ T cells carrying high-affinity TCRs for the WT epitope from those carrying high-affinity ones for the 2F epitope. By using this assay, we found that patients who had been infected with the WT virus first produced WT-specific CD8$^+$ T cells and then 2F-specific CD8$^+$ T cells approximately 6 to 12 months after the 2F mutant had become predominant. In those patients, the 2F mutant virus appeared more than 12 months after the WT virus infection. These findings support our contention that 2F is an escape mutant and that the three donors who had 2F sequences in

FIG. 5. Detection of Nef138-10-2F-specific CTLs in HIV-1-infected patients who had been infected with 2F mutant virus. (A) Tetramer binding of Nef138-10-specific CTLs. KI-158-derived CTL clone 189 (top) and KI-144-derived CTL clone 82 (bottom) were stained with either WT (left panels) or 2F (middle panels) tetramers or both (right panels). The percentage of tetramer-positive cells among CD8$^+$ cells was measured. (B and C) Ex vivo analysis of Nef138-10-specific and Nef138-10-2F-specific CTLs. Nef138-10-specific CTLs in PBMCs derived from HLA-A*2402$^+$ HIV-1-infected individuals were measured by using both WT and mutant (2F) tetramers. Symbols: ●, WT tetramer-positive cells; ○, plasma viral loads. The epitope sequences from viral RNA (plasma) or provirus DNA (PBMCs) during the clinical course are shown. The *x* axis represents weekly course from the first visit. The frequency of CD8$^+$ cells positive for each or both tetramers is given in the quadrants below the graphs. VL, viral load.

their plasma RNA and proviral DNA during the early phase were primarily infected with 2F virus.

In the present study, we demonstrated that new hosts could effectively produce the 2F escape mutant-specific CTLs, even though the 2F mutant epitope was very weakly presented by HLA-A*2402 in HIV-1-infected cells. The 2F-specific CTLs could suppress replication of the 2F mutant virus, but this ability was much weaker toward the 2F mutant than toward the WT virus. The reversion from 2F to WT was not found in the three patients who had been infected primarily with the 2F mutant virus and monitored for 2 to 3 years. This lack of reversion is explained by the fact that the 2F-specific CTLs could effectively suppress replication of the WT virus. This mutant accumulated in HLA-A*2402$^-$ USI patients. Since HLA-A*2402 is a common allele found in approximately 70% of the Japanese population, the 2F mutant can accumulate in the Japanese AIDS population.

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Short Communication

Effects of Low HIV Type 1 Load and Antiretroviral Treatment on IgG-Capture BED-Enzyme Immunoassay

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ABSTRACT

The IgG-capture BED-enzyme immunoassay (BED-CEIA) is used widely at present to detect recent HIV-1 seroconversion. However, antibody levels and antibody kinetics are impacted by HIV-1 load and antiretroviral treatment, which may have a significant effect on the assay results. In this study, we analyzed serial samples from 11 patients with recent infection, including four patients treated by structured treatment interruption (STI), and compared the results with those of 10 untreated and 7 treated patients with chronic infection. The BED-CEIA missidentified one long-term nonprogressor hemophiliac with an extremely low HIV-1 load and five patients with chronic infection who received antiretroviral treatment. We also found that the ODn values increased slowly in patients with recent infection and low HIV-1 loads and that the ODn values fluctuated in parallel with HIV-1 load during STI. Our data indicate that the results of BED-CEIA are influenced by HIV-1 load and antiretroviral treatment. Care should be taken when interpreting the results of BED-CEIA, especially in individuals with low HIV-1 loads. Those on antiretroviral treatment should be excluded from BED-CEIA testing to improve the predictive value of detecting recent infections.

According to the Committee on HIV/AIDS Trends (the Ministry of Health, Labor, and Welfare of the Japanese government), the number of newly diagnosed cases of HIV-1 infection in Japan is continuously increasing and the most frequent mode of transmission is homosexual contact among men who have sex with men.[1] Assessing the incidence of recent infection is important to monitor the current HIV-1 epidemic, although the diagnosis of recent infections usually requires longitudinal follow-up. A new immunoglobulin G (IgG)-capture BED-enzyme immunoassay (BED-CEIA) (Calypte Biomedical Corp., Rockville, MD) was developed recently to identify recent HIV-1 infections.[2,3] BED-CEIA measures the proportion of HIV-specific IgG in serum or plasma samples, which increases after seroconversion. In brief, plates coated with goat antihuman IgG are used to capture both HIV-specific and non-HIV-IgG in test samples. The HIV-specific IgG is detected by a branched multisubtype gp41 peptide labeled with biotin. Incubation with streptavidin-peroxidase followed by tetramethylbenzidine (TMB) substrate allows colorimetric detection of HIV-IgG. The optical density (OD) values of test specimens are normalized (ODn) relative to the value of a calibrator (specimen OD/calibrator OD) to minimize interrun variations. According to the instructions provided by the manufacturer, an ODn of 0.8 corresponds to a mean seroconversion duration of 153 days and the samples with an ODn of <0.8 are considered to be from individuals with recent infection.[4] To assess the reliability of BED-CEIA, we used multiple samples from 28 HIV-1 subtype B-infected patients after obtaining written informed consent.[1]

First, we analyzed the samples of 11 patients with recent infection, 10 untreated patients with chronic infection, and 7 treated patients with chronic infection. The diagnosis of recent infection was made based on the increasing bands of Western blotting against HIV-1 antigens and the used samples were taken at the first visit. The BED-CEIA ODn values of all the 11 samples of recent infection were <0.8 (mean 0.118, SD 0.124) and were correctly identified as recent infection (Fig. 1). Blood samples were also taken from 10 antiretroviral treatment-naive patients with chronic infection (more than 2 years after the first visit). The ODn values of nine of these samples were

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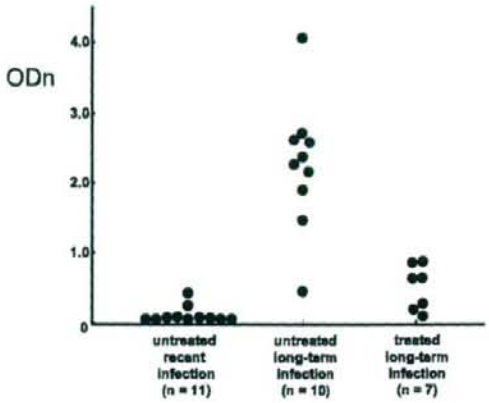

FIG. 1. ODn values of BED-CEIA in three groups of cases. Shown are the ODn values of untreated patients with recent HIV-1 infection and untreated and treated patients with chronic HIV-1 infection.

>1.4 and were correctly identified as long-term infection (mean 2.462, SD 0.718). One sample, however, was missidentified as recently infected (ODn = 0.447), which was taken from a long-term nonprogressor hemophiliac carrying an undetectable HIV-1 load (<50 copies/ml) who had not received antiretroviral treatment and who had acquired his HIV-1 infection before 1985.[5] Blood samples were also taken from seven patients with long-term infection who had received antiretroviral treatment and whose viral load had been persistently suppressed below the detection limit for more than 2 years. Their BED-CEIA ODn values (mean 0.508, SD 0.320) were lower than those of untreated patients with chronic infection ($p = 0.0003$), and five of the seven were incorrectly labeled as recently infected, indicating that antiretroviral treatment negatively alters the reliability of BED-CEIA.

Next, we analyzed serial samples from the same patients to determine the longitudinal changes in ODn. Four of the 11 patients described above with recent infection were subsequently treated with structured treatment interruption (STI), which involves repeated cycles of treatment and interruption intended to evoke a host immune response against HIV-1.[6] We compared the ODn values of these patients with those of the other seven patients who did not receive any treatment to define the natural change after recent infection (Fig. 2). In four patients with logarithmic averages of an HIV-1 load of $\geq 2.0 \times 10^4$ copies/ml, the ODn values gradually increased and all the samples taken more than 153 days after the first visit were correctly identified as long-term infection. However, in the other three patients whose logarithmic averages of HIV-1 load were $\leq 2.8 \times 10^3$ copies/ml (low viral load), the ODn values increased more slowly and many samples were misslabeled as recent infection although they were taken more than 1 year after the first visit. We also analyzed serial samples taken from the treatment-naive long-term nonprogressor hemophiliac described above, and, surprisingly, found a slow increase in the ODn value (Fig. 3). The ODn value of a sample taken in 2005, more than 20 years

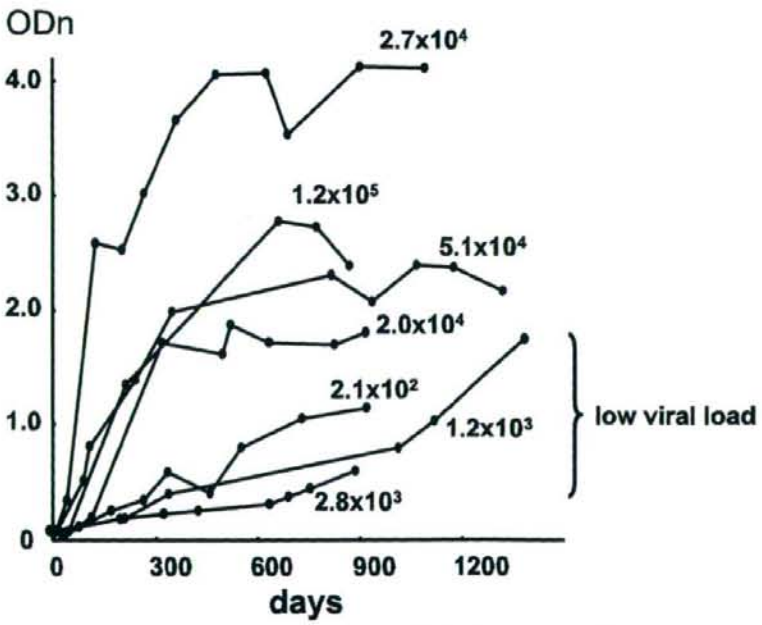

FIG. 2. Serial changes in ODn values of untreated patients with HIV-1 infection. Lines indicate serial changes in ODn values of seven patients with recent infection including three cases with low viral loads.

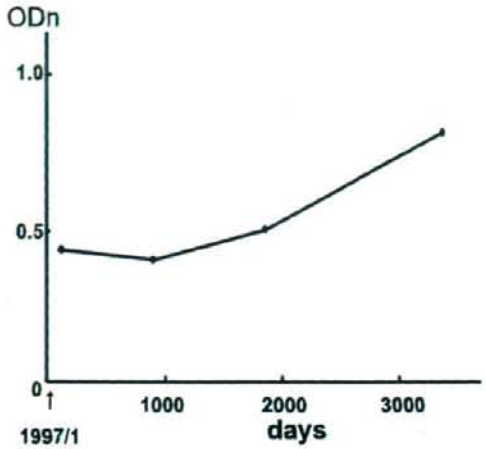

FIG. 3. Serial change in ODn values of one long-term nonprogressor hemophiliac. The line indicates serial changes in ODn values of a long-term nonprogressor with persistently undetectable HIV-1 load who acquired infection before 1985 and had not received antiretroviral treatment. Day 0 is the date of his first visit in January 1997.

after acquiring HIV-1 infection, was 0.865. These data indicate that the ODn values of patients with a low HIV-1 load increase slowly and false-positive recent infection can occur in such cases.

Finally, we analyzed serial samples of four patients with recent infection who received antiretroviral STI therapy to determine the effect of such treatment on ODn values. Figure 4 shows the changes in HIV-1 load and BED-CEIA ODn values of one patient, in whom conventional continuous antiretroviral treatment was administered 462 days after the completion of STI. After the introduction of the first course of antiretroviral treatment, the HIV-1 load sharply decreased and ODn values were persistently low (<0.20). Following a drop in HIV-1 load to below the detection limit (<50 copies/ml), treatment was interrupted for 26 days, during which the HIV-1 load rebounded accompanied by an increase in ODn to 0.760. Then the second course of treatment was introduced, which resulted in a fall of HIV-1 load (400 copies/ml) and ODn value (0.482). The fluctuation in ODn value paralleled the HIV-1 load during STI. Similar data were obtained from the other three cases treated with STI. Considered together, these findings indicate that introduction of antiretroviral treatment resulted in a rapid fall in BED-CEIA ODn values, probably due to antiretroviral treatment-induced suppression of HIV-1 load, suggesting that ODn value are sensitive to changes in HIV-1 load.

Recently, BED-CEIA has been used in a number of cross-sectional populations to estimate incidence and showed excellent results.[3,7] There was plausible agreement between the observed and BED-CEIA-estimated incidence with specimens obtained from a longitudinal cohort study[8] and there was no missclassification of 70 pregnant women with known long-term infection.[3] However, missidentification of long-term infection as recent infection can happen in some cases.[4] Our study indicates that such missidentification is associated with low HIV-1 load and use of antiretroviral treatment, which is consistent with a previous report.[4]

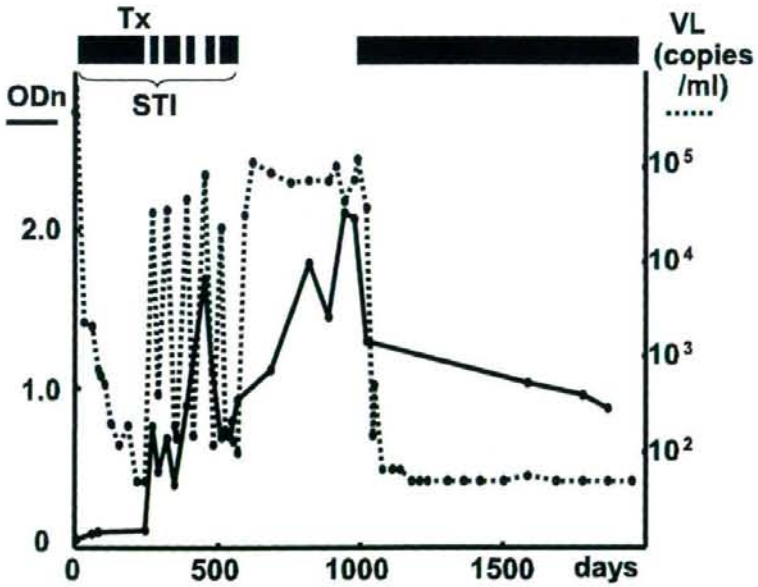

FIG. 4. Effect of antiretroviral treatment on ODn values. Bold and dotted lines show changes in ODn values and HIV-1 load, respectively, in a patient who received STI followed by conventional antiretroviral treatment.

Therefore, whenever possible, those on antiretroviral treatment should be excluded from BED-CEIA testing to improve the predictive value of detecting recent infections.

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REVIEW

Pharmacogenetic information derived from analysis of *HLA* alleles

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A large amount of pharmacogenetic information has, in particular, accumulated on the association between human leukocyte antigen (*HLA*) alleles and hypersensitivity to certain drugs. Prospective *HLA* typing has dramatically reduced the risk of abacavir hypersensitivity because of its strong association with *HLA-B*5701*. Significant predisposition to nevirapine hypersensitivity has been reported in Caucasian Australians harboring *HLA-DRB1*0101* with high CD4+ T-cell counts, and Sardinians and Japanese harboring *HLA-Cw8*. A strong association between carbamazepine hypersensitivity and *HLA-B*1502* has been reported in Han Chinese. Most Han Chinese individuals with allopurinol-induced severe cutaneous adverse reactions are positive for *HLA-B*5801*. *HLA* typing can stratify risk of hypersensitivity to certain drugs and allow personalized treatment, although the patients should be monitored closely even if they are negative for *HLA* alleles associated with hypersensitivity.

Hypersensitivity reactions can occur with most drugs, although their frequency, severity and clinical manifestations vary. They commonly involve the skin and mucosal surfaces, and in severe cases can result in Stevens–Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN). Other severe hypersensitivity reactions can affect other organs such as the liver (hepatitis), lung (pneumonitis) and digestive system (gastrointestinal bleeding), and show more generalized symptoms [1]. Rechallenge with the same drugs usually induces more severe reactions, even fatal reactions in some cases, suggesting that hypersensitivity reactions are immunological memory responses after sensitization. These reactions affect only a minority of patients taking the drug. However, hereditary forms of severe drug hypersensitivity and cases occurring in identical twins have been reported, implying the involvement of certain genetic factors in predisposing individuals to such hypersensitivity reactions [2,3]. Given the immunological basis of their mechanisms, it is not surprising that the associations between human leukocyte antigen (*HLA*) alleles and hypersensitivity to some drugs have been reported during the past decade. HLA is a key molecule in T-cell-mediated immune reactions. It presents antigens (usually eight or nine peptide residues) to T-cell receptors (TCRs), thereby selecting antigen-specific T cells and initiating immune responses. Such reactions usually occur in viral and bacterial infections, and microbe-derived peptides restricted by host HLA are targeted by antigen-specific immune responses [4]. Since drugs and their metabolites are small chemical compounds, they do not usually trigger immune reactions by themselves. However, they may conjugate or bind to intracellular proteins, where they are presented as antigens or haptens by MHC class I or class II molecules to $CD8^+$ or $CD4^+$ T cells, resulting in activation of drug-specific T cells [5,6].

We will review in this article the recent literature on the association between *HLA* allele and hypersensitivity reactions to abacavir, nevirapine, carbamazepine and allopurinol. We will also discuss the clinical implications of such associations, with a special focus on the association of *HLA-B*5701* with hypersensitivity to abacavir, an anti-HIV-1 agent, because it is the most well analyzed and reported. Widespread genetic screening of such association in HIV-1-infected individuals can be used to prevent hypersensitivity reactions.

Abacavir hypersensitivity & *HLA-B*5701*

The currently recommended anti-HIV-1 treatment is the use of a combination regimen. The initial regimen for treatment-naive infected individuals should contain two nucleoside/nucleotide reverse transcriptase inhibitors (NRTI) and either a non-nucleoside reverse transcriptase (NNRTI) or an HIV protease inhibitor [7,101]. The action of the NRTI drug class is to inhibit viral replication through competitive inhibition of viral RNA-dependent DNA polymerase (reverse transcriptase) that allows the creation of a nascent DNA sequence from its own RNA template, whereas NNRTI drugs function by direct binding and inactivation of the polymerase. HIV protease

Keywords: abacavir, allopurinol, carbamazepine, HIV, hypersensitivity, nevirapine

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inhibitors prevent the cleavage of the Gag protein and Gag–Pol protein precursors, thus inhibiting viral replication at a later stage in the replication cycle [8]. NRTIs have been prescribed since the late 1980s, and their advantages and disadvantages are well recognized. A major adverse effect of NRTI is mitochondrial toxicity, which can result in life-threatening lactic acidosis [9–11]. Two recently developed NRTIs, tenofovir disoproxil fumarate (TDF) and abacavir, have low mitochondrial toxicity and both can be prescribed with once-daily dosing [12,13]. However, only TDF is listed as a preferred NRTI in the guideline of the Department of Health and Human Services. On the other hand, abacavir is listed as an alternative NRTI because of its potential for serious hypersensitivity reactions in 5–8% of Caucasians [14,101].

The safety data for abacavir are well described and based on approximately 200,000 patients who received abacavir in clinical trials. The most important limitation to continuous use of this drug is hypersensitivity reactions [15,16]. Such reactions are multi-organ clinical syndromes, which generally occur within the first 6 weeks of abacavir treatment, and typically present with fever, skin rash, malaise/fatigue, gastrointestinal symptoms (e.g., nausea, vomiting and diarrhea) and/or respiratory symptoms (e.g., dyspnea, cough and pharyngitis) [15]. It is important to make a correct diagnosis of abacavir-related hypersensitivity reactions, since a rechallenge with abacavir after an initial reaction can evoke a more rapid reappearance of more severe symptoms within hours of re-exposure, which could result in death in some cases [17–19]. Unfortunately, abacavir hypersensitivity reactions are sometimes difficult to distinguish from systemic viral illness or similar drug reactions caused by other concurrently administered antiretroviral drugs or antibiotics [20].

Meta-analysis of clinical trials indicating a low risk of abacavir hypersensitivity reactions in black people, as well as a case report of familial hypersensitivity, are strong indicators of a genetic basis of this idiosyncratic syndrome [21,22]. Two independent studies identified a strong association between abacavir hypersensitivity and *HLA-B*5701*, which can assist clinicians in predicting those individuals who could develop hypersensitivity reactions and to make a correct diagnosis of hypersensitivity reactions in abacavir-treated individuals, although the association was observed only in Caucasians but not in the black people originally (Table 1) [23,24]. In addition to *HLA-B*5701*, the possession of *HLA-DR7* and *HLA-DQ3*, which are markers of the 57.1 ancestral haplotype, is associated with an increase in the odds ratio of hypersensitivity risk, suggesting that another causative genetic region is linked to *HLA-B*5701* [23]. Fine recombinant genetic mapping has identified a significant linkage disequilibrium of the haplotypic M493T polymorphism of heat shock protein-Hom (Hsp70-Hom; Hsp1AL) and *HLA-B*5701* in abacavir hypersensitive cases, which simplified and enhanced the discrimination of hypersensitive subjects from tolerant controls when compared with the *HLA-B*5701* test alone (Table 1) [25]. The Hsp70-Hom M493T polymorphism may facilitate loading of abacavir- or its metabolite-haptenated endogenous peptides onto *HLA-B*5701* [26]. High intracellular and extracellular levels of TNF are

Table 1. Drug hypersensitivity and associated *HLA* alleles.

Study	Drug	HLA	Population	OR	Pc	Ref.
Mallal *et al.* (2002)	Abacavir	*B*5701*	Australian	117	$<10^{-4}$	[23]
Hetherington *et al.* (2002)	Abacavir	*B*5701*	British	24	$<10^{-4}$	[24]
Martin *et al.* (2004)	Abacavir	*B*5701*	Australian	960	$<10^{-4}$	[25]
Martin *et al.* (2005)	Nevirapine	*DRB1*0101* and high CD4	Caucasian Australian	18	0.0006	[58]
Littera *et al.* (2006)	Nevirapine	*Cw8-B14(65)*[‡]	Sardinian	15	0.05	[59]
Gatanaga *et al.* (2007)	Nevirapine	*Cw8*	Japanese	6.2	0.03	[60]
Chung *et al.* (2004)	Carbamazepine	*B*1502*	Han Chinese	2504	$<10^{-4}$	[68]
Hung *et al.* (2006)	Carbamazepine	*B*1502*	Han Chinese	1357	$<10^{-4}$	[69]
Hung *et al.* (2005)	Allopurinol	*B*5801*	Han Chinese	580	$<10^{-4}$	[75]

[‡]Cw*0802 and B*1402 are in strong linkage equilibrium in Sardinians.

present in abacavir-stimulated peripheral blood mononuclear cells (PBMCs) of abacavir-hypersensitive patients, relative to those of abacavir-tolerant individuals, and depletion of $CD8^+$ T cells results in reduction of TNF levels [25]. Considering that marked infiltration of $CD8^+$ T cells is observed in cutaneous abacavir patch testing of hypersensitive patients and that higher $CD8^+$ T-cell count is a risk factor of hypersensitivity reactions, *HLA-B*5701*-restricted $CD8^+$ T cells must play a major pathogenic role in abacavir hypersensitivity reactions [27–29].

Prospective *HLA-B*5701* genetic screening has been instituted in clinical practice in Western Australia, the UK and Paris for abacavir-naive patients, and this had markedly reduced the risk of developing abacavir hypersensitivity (Table 2) [30–32]. This strategy unexpectedly reduced the proportion of patients who stopped their treatment after the appearance of symptoms that were otherwise unrelated to hypersensitivity reactions, suggesting that genetic screening seems to prevent overestimation of hypersensitivity reactions with subsequent discontinuation of abacavir in *HLA-B*5701*-negative individuals [30,32]. The PREDICT-1 study randomized patients either to receive abacavir according to standard of care or to be prospectively screened for *HLA-B*5701* before starting abacavir (to exclude *HLA-B*5701* carriers) [33]. The incidence of hypersensitivity reactions was significantly lower in the prospective screening arm compared with the control arm. However, most of the screened patients described above were Caucasian, and the utility and cost–effectiveness of the genetic screening largely depends on the prevalence of *HLA-B*5701* in the targeted population [34]. The prevalence of *HLA-B*5701* among Hispanics and black people is lower than Caucasians, and the relationship between *HLA-B*5701* and abacavir hypersensitivity was described as weak in Hispanics and nonexistent in black patients [35,36]. The SHAPE study corroborated the low rate of abacavir hypersensitivity immunologically confirmed by skin patch testing in black patients, but it also reported high sensitivity of *HLA-B*5701* in immunologically validated cases in both whites and blacks, suggesting the importance of supplementing a clinical definition of abacavir hypersensitivity by immunological assessment [37]. In our study, none of the 669 Japanese HIV-1-infected patients had *HLA-B*5701*, yet hypersensitivity reactions occurred in seven (all *HLA-B*5701*-negative, not immunologically confirmed) of 536 Japanese patients exposed to abacavir [38]. Thus, genetic screening of *HLA-B*5701* does not seem cost-effective in Japanese populations. Close monitoring of patients after abacavir prescription without HLA typing may be a more reasonable approach in the populations that do not carry *HLA-B*5701*.

Interestingly, strong reponses of *HLA-B*57*-restricted cytotoxic T lymphocytes can ocur against multiple HIV-1 epitopes, which is considered to result in slow disease progression of *HLA-B*57*-positive HIV-1-infected individuals [39,40]. One of the major *HLA-B*57*-restricted epitopes is located in codons 244–252 of HIV-1 reverse transcriptase, which is routinely sequenced as a part of drug-resistance testing [7,41,101]. Furthermore, cytotoxic T lymphocytes escape mutations (wild-type V to E, M and L) are commonly observed at codon 245 in *HLA-B*57*-positive patients, which may serve as an indirect marker for the presence of *HLA-B*5701* [40,42]. In one study [43], the negative predictive value was over 99% (meaning that the presence of wild-type amino acid V at codon 245

Table 2. Reduced frequencies of abacavir hypersensitivity reactions after *HLA-B*5701* genetic screening.

Study	Country	n (%)‡		p-value	Ref.
		Before screening	*After screening*		
Rauch *et al.* (2006)	Australia	16/199 (8.0)	3§/151 (2.0)	0.01	[30]
Reeves *et al.* (2006)	UK	20/321 (6.2)	1¶/155 (0.6)	0.002	[31]
Zucman *et al.* (2007)	France	11#/49 (22.4)	0/128 (0)	$<10^{-4}$	[32]

‡*Number (%) of hypersensitive patients/abacavir-treated patients.*

§*All three individuals were* HLA-B*5701 *positive; two inadvertently exposed to abacavir because of a lack of review of HLA results, and one on the basis of his own content.*

¶HLA-B*5701 *negative; non-HIV-expert physician discontinued therapy because of possible hypersensitivity reactions.*

#*Included five* HLA-B*5701 *negative cases of possible hypersensitivity based on wide-range clinical criteria.*

excludes the possibility of *HLA-B*5701* in >99% of cases), while the positive predictive value was low (20%). These results suggest that abacavir can be safely prescribed to most HIV-1-infected patients harboring wild-type V at codon 245 in reverse transcriptase [43]. This method can save the cost of HLA typing by utilizing the HIV-1 sequence data, which are obtained from routine resistance testing approved by the public and private health insurance industries of many developed countries. However, it may result in inadequate withholding of abacavir in a significant number of *HLA-B*5701*-negative patients infected with escape HIV-1 variants, because these escape mutations are often observed and probably able to persist over long periods even in the absence of *HLA-B*5701*-restricted cytotoxic T lymphocyte pressure. Another problem is differences among HIV-1 subtypes. The wild-type amino acid at codon 245 in reverse transcriptase is V only in HIV-1 subtype B, which is most prevalent in developed countries, but is another amino acid such as Q or E in non-B subtypes. Therefore, this method is not suitable when the obtained HIV-1 sequence in phylogenetic analysis belongs to non-B subtypes, which decreases its utility in African and Asian countries where non-B subtypes are prevalent. Considering that practical and accurate HLA typing has already been implemented and is effectively identifying *HLA-B*5701* carriers [44], direct HLA typing is a more simple and better approach to stratify the risk of abacavir hypersensitivity than speculating HLA type from HIV-1 sequences.

Nevirapine hypersensitivity & associated *HLA* alleles

Nevirapine is also a well-tolerated anti-HIV-1 agent, which is listed as an alternative NNRTI in the HIV-1 treatment guideline of the Department of Health and Human Services [45,101]. The most common adverse event associated with the use of nevirapine is hypersensitive reactions (observed in 4.9% of recipients), which are characterized by a combination of rash, fever or hepatitis, and typically occurs within the first 6 weeks of initiation of treatment and can be more rapid and severe with re-challenge [46,47]. Woman with high $CD4^+$ T-cell counts appear to be at higher risk of hypersensitivity reactions [48,49]. The HIV-1 treatment guidelines do not recommend the use of nevirapine for female patients with $CD4^+$ T cell counts over 250 cells/mm^3 and male patients with $CD4^+$ T-cell counts over 400 cells/mm^3 [7,50–53,101]. A higher incidence of hypersensitivity reactions was reported in non-HIV-infected individuals who received nevirapine as part of post-exposure prophylactic treatment, probably associated with a high CD4 count [54]. Usually, cutaneous diseases, including drug hypersensitivity to sulfamethoxazole, dapsone and antituberculous agents, are extremely common in patients with HIV infection, and their incidence increases as immune function deteriorates [55]. However, conversely, in the case of nevirapine hypersensitivity, normal and relatively maintained immune function is a risk factor for unknown reasons [56].

The description of nevirapine-induced SJS in a Ugandan mother and her son suggests a genetic basis for nevirapine hypersensitivity [57]. The possession of *HLA-DRB1*0101* is associated with increased risk of nevirapine hypersensitivity involving multisystemic or hepatotoxic reactions, and which was abrogated by low $CD4^+$ T-cell counts, in the Western Australian HIV Cohort (**Table 1**) [58]. Littera *et al.* reported that the *HLA-Cw*0802-B*1402* haplotype is associated with nevirapine hypersensitivity in Sardinian patients [59]. We also reported a significant association between *HLA-Cw8* and nevirapine hypersensitivity in Japanese patients, suggesting that nevirapine or its metabolite coupled with *HLA-Cw8* antigen may be expressed on the cell surface and may induce hypersensitivity reactions (**Table 1**) [60]. In this regard, there was no significant association between *HLA-DRB1*0101* and hypersensitivity in the Sardinian and Japanese cohorts described above, implying that primarily determining HLA alleles may be different among populations. Isolated mild rash and simple hepatotoxicity often occur within 6 weeks of nevirapine treatment initiation. It is possible that this reaction is pathologically different from the severe hypersensitivity reactions, making the definition of hypersensitivity confusing and comparison of different studies difficult [58,61,62]. Establishment of a standardized definition and accurate diagnosis of hypersensitivity seems indispensable for further study of the linkage between HLA alleles and nevirapine hypersensitivity.

Carbamazepine-induced SJS/TEN & *HLA-B*1502*

Carbamazepine is one of the most widely used anticonvulsants, and is also used in bipolar depression and trigeminal neuralgia. Carbamazepine is generally well tolerated but can cause dose-dependent adverse reactions such as dizziness and nystagmus [63]. It is also associated with idiosyncratic hypersensitivity reactions, most

commonly skin rashes such as SJS and TEN, accompanied with fever, lymphadenopathy, and multiorgan-sysem abnormalities [64]. A high frequency of carbamazepine-related hypersensitivity reactions was reported in South-East Asian countries compared with 0.01–0.1% in Caucasians [64–67]. Furthermore, carbamazepine hypersensitivity was reported in identical twins [3]. These studies suggest that susceptibility to such reactions may be genetically determined.

A Taiwanese study reported a strong association between carbamazepine-induced SJS/TEN and the *HLA-B*1502* allele in Han Chinese [68]. The finding was confirmed later by the same group in another study that included patients who were Han Chinese or Chinese descendants from Taiwan, Hong Kong, China and the USA (Table 1) [69]. The allele frequency of *HLA-B*1502* is 3–12% in South-East Asians and less than 0.1% in Caucasians, which may explain the higher incidence of carbamazepine-induced SJS/TEN in South-East Asia. In one European study, 15 patients with carbamazepine-induced SJS/TEN were analyzed and five patients who had a parent of Asian origin were positive for the *HLA-B*1502* allele. The remaining ten patients, who were Caucasians, were *HLA-B*1502*-negative [70]. Another European study of Caucasians did not find any *HLA-B*1502*-positive patients who were hypersensitive to carbamazepine [71]. Considered together, *HLA-B*1502* does not seem to be associated with carbamazepine hypersensitivity in the Caucasian population and ethnicity seems important. While it seems conceivable that the causative genetic region of carbamazepine hypersensitivity is linked to *HLA-B*1502*, especially in the Han Chinese population, fine recombinant genetic mapping confirmed the susceptibility gene is *HLA-B*1502* itself [69].

Allopurinol-induced severe cutaneous adverse reactions & *HLA-B*5801*

Allopurinol is widely used for hyperuricemia and recurrent urate kidney stones [72]. However, it is also one of the most frequent causes of severe cutaneous adverse reactions including SJS and TEN [73]. Familial predisposition has been reported and susceptibility to such idiosyncratic reactions is thought to be genetically determined [74]. One Taiwanese study reported a strong association between allopurinol hypersensitivity and *HLA-B*5801* in a Han Chinese population and recombinant genetic mapping further identified *HLA-B*5801* itself as the major susceptibility gene (Table 1) [75]. In support of these results, a Japanese group reported three cases with different manifestations of allopurinol hypersensitivity and all of them were positive for *HLA-B*58* [76].

Conclusion

We reviewed here the HLA association with hypersensitivity to abacavir, nevirapine, carbamazepine and allopurinol. Considering that hypersensitivity reactions to abacavir can be life-threatening and even fatal, abacavir prescription to *HLA-B*5701* should be avoided. The following prescriptions should be followed by close monitoring of the patients: nevirapine to patients positive for *HLA-DRB1*0101* or *Cw8*, carbamazepine to *HLA-B*1502* holders and allopurinol to *HLA-B*5801*-positive patients, even if the patient is from a population with no described allele association, because one cannot exclude possible association. It is noteworthy that pharmacogenetic studies are more likely to yield negative results when conducted in populations with low frequencies of the possibly associated allele [77]. More importantly, patients treated with any of these drugs should be monitored closely even if they are negative for *HLA* alleles that are known to be associated with hypersensitivity. Hypersensitivity reactions can potentially occur in any patient as they may hold *HLA* alleles that have yet unreported associations with hypersensitivity. Application of genetic screening should not substitute appropriate clinical vigilance and patient management.

Before abacavir-containing treatment is introduced for HIV-infected patients, HLA analysis should be performed to exclude *HLA-B*5701*, unless the patient is from a population which does not carry *HLA-B*5701*. Such exclusion of *HLA-B*5701* would markedly reduce the possibility of hypersensitivity reactions and prevent overestimation of hypersensitive reaction that could otherwise result in excessive discontinuation of treatment [29–31].

HLA associations with nevirapine hypersensitivity have been reported, but the odds ratios are not high [58–60]. According to the HIV-1 treatment guidelines, avoiding nevirapine prescription is reasonable for female patients with $CD4^+$ T-cell counts over 250 cells/mm^3 and male patients with $CD4^+$ T-cell counts over 400 cells/mm^3, without HLA typing [7,53,101].

Strong associations between carbamazepine hypersensitivity and *HLA-B*1502*, and between allopurinol hypersensitivity and *HLA-B*5801* have been reported in Han Chinese population [68,69,75].

Analysis of these associations in different ethnic populations is urgently needed before it is widely applied in clinical practice.

Future perspective

Current pharmacogenetic information is limited in relation to the genes of HLA, metabolizing enzymes and drug transfer proteins. Considering that the technology to identify genetic variants across the whole genome is advancing rapidly, many more significant genetic factors for drug efficacy and adverse reactions are likely to be identified in the future. Identification of such factors is important not only to discover new pharmacological mechanisms, but also to improve the currently available drugs and to develop novel drugs. In such whole-genome analysis, drug-induced phenotypes should be carefully observed in genetically variable populations, which will be feasible only through international collaboration.

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Executive summary

- Human leukocyte antigen (HLA) information can help predict risk of some drug hypersensitivity.

Abacavir hypersensitivity & HLA-B*5701

- Abacavir hypersensitivity is strongly associated with *HLA-B*5701*.
- Prospective HLA screening can markedly reduce the risk of abacavir hypersensitivity.

Nevirapine hypersensitivity & associated HLA alleles

- Significant predisposition to nevirapine hypersensitivity has been reported in Caucasian Australians harboring *HLA-DRB1*0101* with high CD4+ T-cell counts, and Sardinians and Japanese harboring *HLA-Cw8*.

Carbamazepine-induced SJS/TEN & HLA-B*1502

- Carbamazepine hypersensitivity is frequent in *HLA-B*1502*-positive Han Chinese.

Allopurinol-induced severe cutaneous adverse reactions & HLA-B*5801

- Most Han Chinese individuals with allopurinol-induced severe cutaneous adverse reactions are positive for *HLA-B*5801*.

Conclusion

- Prospective HLA screening can stratify the risk of hypersensitivity to abacavir, nevirapine, carbamazepine and allopurinol, and allows personalized medicine.
- Application of genetic screening should not substitute appropriate clinical vigilance and patient management.

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Amino Acid Mutation N348I in the Connection Subdomain of Human Immunodeficiency Virus Type 1 Reverse Transcriptase Confers Multiclass Resistance to Nucleoside and Nonnucleoside Reverse Transcriptase Inhibitors▽†

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We identified clinical isolates with phenotypic resistance to nevirapine (NVP) in the absence of known nonnucleoside reverse transcriptase inhibitor (NNRTI) mutations. This resistance is caused by N348I, a mutation at the connection subdomain of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT). Virologic analysis showed that N348I conferred multiclass resistance to NNRTIs (NVP and delavirdine) and to nucleoside reverse transcriptase inhibitors (zidovudine [AZT] and didanosine [ddI]). N348I impaired HIV-1 replication in a cell-type-dependent manner. Acquisition of N348I was frequently observed in AZT- and/or ddI-containing therapy (12.5%; $n = 48$; $P < 0.0001$) and was accompanied with thymidine analogue-associated mutations, e.g., T215Y ($n = 5/6$) and the lamivudine resistance mutation M184V ($n = 1/6$) in a Japanese cohort. Molecular modeling analysis shows that residue 348 is proximal to the NNRTI-binding pocket and to a flexible hinge region at the base of the p66 thumb that may be affected by the N348I mutation. Our results further highlight the role of connection subdomain residues in drug resistance.

Combinations of multiple drugs used for clinical treatment of human immunodeficiency virus type 1 (HIV-1) infections in highly active antiretroviral therapies (HAART) can dramatically reduce viral load, increase levels of CD4positive cells, improve survival rates, and delay the onset of AIDS. HAART typically includes two nucleoside reverse transcriptase inhibitors (NRTIs) and a nonnucleoside reverse transcriptase inhibitor (NNRTI) or a protease inhibitor (17). After prolonged therapy, however, an increasing number of treatment failures are caused by the emergence of multidrug-resistant (MDR) variants. For example, treatment with zidovudine (AZT) and dideoxynuleoside RT inhibitors such as didanosine (ddI) may result in the "Q151 complex" of clinical mutations in RT (A62V/V751/F77L/F116Y/Q151M) which causes high-level resistance to multiple NRTIs, AZT, ddI, zalcitabine (ddC), and stavudine (d4T) (21, 38). Another MDR complex of RT mutations is the "fingers insertion" complex that includes an insertion of two residues at the fingers subdomain of the p66 subunit of RT in the presence of AZT resistance mutations, e.g., M41L and T215Y (M41L/T69SSG/T215Y). This complex can emerge during combination treatment that includes NRTIs (10, 41) and confers resistance to multiple drugs by enhancing the excision reaction that causes resistance by unblocking NRTI-terminated primers (40). G333E or G333D polymorphisms with thymidine analogue-associated mutations (TAMs) and M184V have also been reported to facilitate moderate resistance to at least two NRTIs, AZT and lamivudine (3TC) (7, 22). RT mutations K103N, V106M, and Y188L are associated with resistance to multiple NNRTIs (1, 5). Since all NNRTIs bind at the same hydrophobic binding pocket, mutations in the binding pocket may result in broad cross-resistance between members of this family of drugs.

The presence of variants that are resistant to multiple drugs limits significantly the available therapeutic strategies and, even more profoundly, therapeutic options. However, so far all reports of viruses that acquire resistance to members of both families of RT inhibitors describe variants with multiple mutations at several residues that confer either NRTI or NNRTI resistance. Recently, Paolucci et al. reported that Q145M/L mutations confer cross-resistance to some NRTIs and NNRTIs (31, 32). Similarly, an NNRTI resistance mutation, Y181I, also confers resistance to d4T at the enzyme level (2). The frequency of these mutations in clinical isolates does not appear to be significant, according to the Stanford HIV resistance database (http://hivdb.stanford.edu/index.html); there is no deposition for Q145M/L, and Y181I has a prevalence of 0.02% in drug-naïve or NRTI-treated patients and 0.9% in NNRTI-treated patients.

We report here that N348I is a multiclass resistance mutation involved in resistance to both NRTIs and NNRTIs and present in a significant number of clinical isolates. Residue 348 is at the RT connection subdomain outside the region usually

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sequenced as the drug resistance assay in clinical settings. The role of connection subdomain mutations in AZT resistance has been highlighted recently by Pathak and colleagues (28). The present work shows that N348I confers resistance not only to the NRTI AZT but also to another NRTI, ddI, and two NNRTIs, nevirapine (NVP) and delavirdine (DLV). Importantly, we show that the N348I variant emerges frequently during chemotherapy containing AZT and/or ddI. To our knowledge, this is the first example of a clinically significant and high-prevalence multiclass RTI resistance mutation that highlights the need for extensive phenotypic and genotypic assays to detect novel mutations with important implications on future therapeutic strategies.

MATERIALS AND METHODS

Reagents and cells. AZT, ddI, ddC, and d4T were purchased from Sigma (St. Louis, MO). 3TC, DLV, and tenofovir (TDF) were purchased from Moravek Biochemicals, Inc. (Brea, CA). NVP, abacavir (ABC), and efavirenz (EFV) were generously provided by Boehringer Ingelheim Pharmaceutics Inc. (Ridgefield, CT), GlaxoSmithKline (Philadelphia, PA), and Merck Co. Inc. (Rahway, NJ), respectively. Loviride was kindly provided by S. Shigeta, Fukushima Medical University (Fukushima, Japan). MT-2, SupT1, PM1, H9, Cos-7, and MAGIC-5 cells (CCR5-transduced HeLa-CD4/LTR-β-Gal cells) were cultured and used as described previously (14). Peripheral blood mononuclear cells (PBMCs) obtained from healthy donors were stimulated with phytohemagglutinin (PHA) for 3 days and grown in RPMI 1640 medium with 10% fetal calf serum and 10 U of interleukin-2 as described previously (15, 23).

Clinical isolates. Clinical isolates were obtained from fresh plasma of an HIV-1-infected patient attending the outpatient clinic of the AIDS Clinical Center, International Medical Center of Japan, using MAGIC-5 cells. The isolates were stored at −80°C until use, and infectivity was measured as blue cell-forming units (BFU) of MAGIC-5 cells. The Institutional Review Board approved this study (IMCJ-H13-80), and written informed consent was obtained from the patient.

Viruses and construction of recombinant HIV-1 clones. An HIV-1 infectious clone, pNL101, was kindly provided by K.-T. Jeang (NIH, Bethesda, MD) and used for generating recombinant HIV-1 clones (15). A wild-type (WT) HIV-1, designated HIV-1_{WT}, was constructed by replacing the *pol*-coding region (nucleotides [nt] 2006 of ApaI site to 5785 of SalI site of pNL101) with the HIV-1 BH10 strain. The *pol*-coding region contains a silent mutation at nt 4232 (TTTAGA to TCTAGA; mutation is italicized) for generation of an XbaI unique site. The DNA fragments amplified by reverse transcription-PCR from the primary isolates were digested with appropriate restriction enzymes and cloned into pNL-RT_{WT}. The nucleoside sequences of the PCR-amplified fragments were verified with a model 3730 automated DNA Sequencer (Applied Biosystems, Foster, CA). Viral stocks were obtained by transfection of each molecular clone into Cos-7 cells, harvested, and stored at −80°C until use.

Sequencing analysis of HIV-1 RT region. Viral RNA was extracted from plasma and/or culture supernatant of clinical isolates and subjected to reverse transcription-PCR using a OneStep RNA PCR Kit (Takara Bio, Otsu, Japan). Nested PCR was subsequently conducted for direct sequencing. Primer pairs used for amplification of the DNA fragment from nt 2574 to 3333 of pNL101 were T1 (5′-AGGGGGAATTGGAGGTTT; nt 2393 to 2410) and T4 (5′-TTCT GTTAGTGCTTTGGTT; nt 3422 to 3404) for the first PCR and T12 (5′-CCAG TAAAATTAAAGCCAG; nt 2574 to 2592) and T15 (5′-TCCCACTAACTTCT GTATGTC; nt 3335 to 3315) for the second PCR (15). Primer pairs used for amplification of DNA fragment from nt 3288 to 4316 were 3244F (5′-AT GAACTCCATCCTGACAAATG; nt 3244 to 3265) and 4428R (5′-TGTA CAATCTAATTGCCATAT; nt 4428 to 4407) for the first PCR and 3288F (5′-CCAGAAAAAGACAGCTGGACT; nt 3288 to 3308) and 4316R (5′-TG GCAGATTAAAATCACTAGCC; nt 4316 to 4295) for the second PCR (13). The nested PCR products were then subjected to the direct sequencing of the entire RT coding region, and some PCR products were further analyzed with clonal sequence determination as described previously (13, 15).

Drug susceptibility assay. HIV-1 sensitivity to various RTIs was determined in triplicate using MAGIC-5 cells as described previously (14). MAGIC-5 cells were infected with diluted virus stock (100 BFU) in the presence of increasing concentrations of RTIs, cultured for 48 h, fixed, and stained with X-Gal (5-bromo-4-chloro-3-indolyl-βD-galactopyranoside). The stained cells were counted under a light microscope. Drug concentrations reducing the cell number to 50% of that of the drug-free control (EC_{50}) were determined by referring to the dose-response curve.

Competition assay of HIV-1 replication. MT-2, SupT1, PM1, and H9 cells (2.5×10^5 cells/5 ml) and PHA-stimulated PBMCs (2.5×10^6 cells/5 ml) were infected with each virus preparation (500 BFU) for 4 h. The infected cells were then washed and cultured in a final volume of 5 ml. Culture supernatants (100 μl) were harvested from days 1 to 7 after infection, and the p24 antigen amounts were quantified (27).

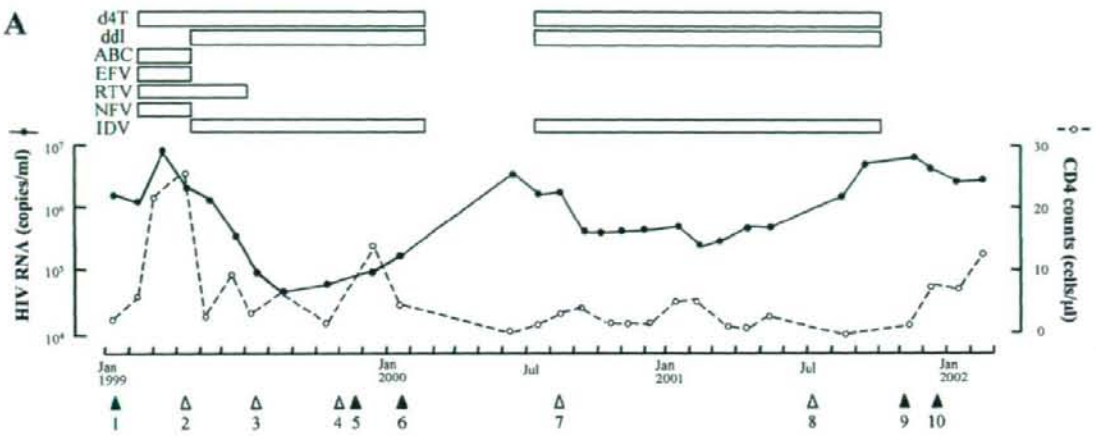
Freshly prepared H9 cells (3×10^5 cells/well) were exposed to the mixture of viral preparations (300 BFU) and cultured to compare their replicative capacities, as previously described (15). On day 1 in culture, one-third of the infected H9 cells were harvested and washed twice with phosphate-buffered saline, followed by DNA extraction. Purified DNA was subjected to nested PCR to sequence the HIV-1 RT genes. The supernatant of the viral culture was transferred to uninfected H9 cells at 7-day intervals, and the cells harvested at each passage were subjected to direct DNA sequencing of the HIV-1 RT gene. Population change of the viral mixture was determined by the relative peak height on the sequencing electrogram. The persistence of the original amino acid substitution was confirmed in all infectious clones used in this assay.

Molecular modeling studies. The SYBYL and O programs were used to prepare molecular models of the complexes of WT and N348I HIV-1 RT with DNA, NVP, and the triphosphates of AZT and ddI. Starting atomic coordinates of HIV-1 RT in complex with DNA were obtained from the structures described by Tuske et al. (40), Sarafianos et al. (36), and Huang et al. (20) (Protein Data Bank [PDB] code numbers 1T05, 1N6Q, and 1RTD, respectively). Because there is no available structure of RT in complex with both NNRTI and DNA, we used structures of RT in complex with NNRTI to obtain initial coordinates of the NNRTI-binding pocket (9, 12). Specifically, we used the coordinates of the two β-sheets of the polymerase active site (β6-β9-β10 that contains the three catalytic aspartates and the YMDD motif as well as β12-β13 of the primer grip) to replace the corresponding regions in the RT-DNA complex. The N348I side chain mutation was manually modeled in the p66 subunit, and all structures were optimized using energy minimization protocols in SYBYL. The triphosphates of AZT and ddI were built based on the structures of AZT monophosphate and dTTP in PDB 1N6Q (36) and 1RTD (20) or of TDF diphosphate in the ternary complex of HIV-1 RT/DNA/TFV-DP, PDB 1T03 (40). The coordinate vector of the resulting structures was varied using a minimization procedure to minimize the potential energy by relieving short interatomic distances while maintaining structural integrity.

RESULTS

Resistance to NNRTIs observed in HIV-1 isolates. The clinical history of the patient is summarized in Fig. 1 and includes the variation of genotypic and phenotypic drug resistance profiles of sequential isolates with time (see also Table S1 and Fig. S1 in the supplemental material). In spite of the combination therapy, little immunologic and virologic response was observed; at time point 2, the CD4 count was 25/μl, and the plasma HIV-1 RNA levels were 2.1×10^6 copies/ml. However, no known drug resistance mutations associated to both NRTIs and NNRTIs were detected in the RT region at this point (Fig. 1B). Due to poor adherence, upon changing the regimen we observed only partial suppression of viral replication and limited increase in the CD4 count. TAMs with N348I accumulated during time points 3 to 6 (Fig. 1). In February 2000, the treatment was interrupted due to severe adverse effects, resulting in a rebound of viral load. In July 2000, the same therapy was resumed for approximately 1 year. No drug resistance-associated mutations were detected upon initiation of this therapy (time point 7). At time point 8, mixtures of two amino acid insertions at codon 69 with TAMs and N348I were detected, although these mutations disappeared after the treatment interruption at time point 10.

Interestingly, HIV-1 isolates at time points 5 and 6 showed resistance to NVP (44- and 25-fold, respectively) and to DLV



B

Time point	Resistance-associated and unique mutations											Fold increase in EC_{50}								
	M41	D67	T69	K70	V75	V118	L210	T215	L283	N348	I393	AZT	ddI	ddC	d4T	3TC	ABC	NVP	DLV	EFV
1	-	-	-	-	-	-	-	-	I	-	L	2	1.5	0.6	1.0	0.9	0.8	2.2	1	1
2	-	-	-	-	-	-	-	-	I	-	L					N.D.				
3	M/L	-	-	-	-	-	-	-	I	-	L					N.D.				
4	L	N	-	-	M	-	W	Y	I	N/I	L					N.D.				
5	L	N	-	-	M	-	W	Y	I	I	L	**44**	**4.3**	0.6	1.8	1.6	1.6	**44**	**8.5**	**3**
6	L	N	-	-	M	I	W	Y	I	I	L	**275**	**5.9**	2.5	**3.2**	**3.4**	2	**25**	**12**	1
7	-	-	-	-	-	-	-	-	I	-	L					N.D.				
8	L	T/N	-/SSG	E	M	I	W	Y	I	N/I	L					N.D.				
9	-	-	-	-	V/M	V/I	L/W	T/Y	I	-	-	2	1.2	0.6	1.1	0.6	1	2.4	1.5	0.7
10	-	-	-	-	-	-	-	-	I	-	-	2.8	0.7	0.6	1	0.8	1.2	1.8	1	1

FIG. 1. The course of patient and drug resistance profiles of clinical isolates obtained from the patient. (A) The drug treatment history is indicated at the top of the graph. The virologic responses represented by plasma viral load and CD4 counts of peripheral blood are shown. Open triangles indicate the time points of genotypic assays. Closed triangles indicate the time points of isolation of clinical isolates for genotypic assays (also see Fig. S1 in the supplemental material) and phenotypic assays (also see Table S1 in the supplemental material showing actual EC_{50} values as mean values and standard deviations from three independent experiments). From February to June 2000 and after October 2001, the chemotherapy was interrupted due to severe adverse effects. (B) The viruses acquired NRTI resistance mutations sequentially as shown. Susceptibility to compounds tested in at least three independent experiments is shown as the relative increase in the EC_{50} compared to HIV-1_{WT} obtained from a pNL4-3-based plasmid. An increase larger than 3.0-fold is indicated in bold. NRTI or NNRTI resistance mutations were reported in the HIV drug resistance database maintained by International AIDS Society 2006, the Stanford University (Stanford, CA) and Los Alamos National Laboratory (Los Alamos, NM), http://hivdb.stanford.edu/and http://resdb.lanl.gov/Resist_DB/, respectively. RTV, ritonavir; NFV, nelfinavir; IDV, indinavir.

(8.5- and 12-fold, respectively) but lacked any known NNRTI resistance-associated mutations except for L283I, which influences susceptibility of NNRTIs when combined with I135L/M/T (6) (Fig. 1B). However, L283I was detected at all points without I135L/M/T even in phenotypically sensitive viruses; therefore, it is unlikely that this single mutation is involved in the resistance. After the interruption at time points 9 and 10, the majority of HIV-1 detected in the plasma reverted to WT and was susceptible to all RTIs tested. The patient was previously treated with a regimen containing EFV, not NVP, for several months prior to the appearance of the N348I mutation. Importantly, this mutation was not detected in genotypic assays during treatment with EFV, but it was first detected 6 months after removal of EFV and use of ddI in the following regimen. Phenotypic and genotypic information at time point 5 shows that resistance to NVP and DLV was present while the patient was on a regimen that did not include any NNRTIs and in the absence of any known NNRTI resistance-related mutations. Thus, it is unlikely that the phenotypically identified NNRTI resistance in the patient was induced by the previous EFV-containing therapy.

RT C-terminal region confers NVP resistance. To identify the mutation(s) responsible for the resistance to NVP and DLV, we constructed chimeric clones with cDNA fragments of the RT region derived from the clinical isolates. Briefly, the N-terminal (amino acids 15 to 267) and C-terminal (amino acids 268 to 560) RT coding regions of clinical isolates were PCR amplified separately and used for replacement of the corresponding regions in the WT sequence of pNL-RT_{WT}. These chimeric clones were then examined for their susceptibility to RTIs (Table 1). Only the clones containing the C-terminal region derived from CL-6 isolated at time point 6 and

TABLE 1. Susceptibility of chimeric HIV-1 clones with N- and/or C-terminal RT region substitutions

RT-replaced region		EC_{50} (fold increase)[a]				
N terminus[b]	C terminus[c]	AZT	ddI	NVP	DLV	EFV
WT[d]	WT	0.038 ± 0.012	2.6 ± 1.04	0.05 ± 0.01	0.03 ± 0.01	0.003 ± 0.001
CL-6[e]	CL-6[e]	3.37 ± 0.97 (**89**)	14.3 ± 0.58 (**5.5**)	1.2 ± 0.21 (**24**)	0.16 ± 0.02 (**5.3**)	0.007 ± 0.004 (2.3)
CL-9[f]	CL-9[f]	0.04 ± 0.01 (1.1)	2.3 ± 1.21 (0.9)	0.13 ± 0.07 (2.6)	0.06 ± 0.02 (2)	0.004 ± 0.002 (1.3)
CL-6	WT	1.24 ± 0.34 (**33**)	4.6 ± 1.50 (1.8)	0.12 ± 0.06 (2)	0.04 ± 0.02 (1.3)	0.002 ± 0.001 (0.7)
WT	CL-6	0.19 ± 0.04 (**5**)	13.7 ± 2.31 (**5.3**)	1.67 ± 0.23 (**33**)	0.39 ± 0.06 (**13**)	0.006 ± 0.002 (2)
CL-6	CL-9	1.50 ± 0.95 (**39**)	5.9 ± 1.21 (2.3)	0.10 ± 0.05 (2)	0.04 ± 0.02 (1.3)	0.002 ± 0.001 (0.7)

[a] The data shown are mean values ± standard deviations obtained from the results of at least three independent experiments, and the relative increase in the EC_{50} values for recombinant viruses compared with WT is shown in parentheses. Bold indicates an increase in EC_{50} value greater than threefold relative to the WT.
[b] RT N-terminal region contains mainly the domains of finger and palm and partially thumb (amino acid positions 15 to 267).
[c] RT C-terminal region contains domains of thumb, connection, and RNase H (amino acid positions 268 to 560).
[d] DNA fragment is identical to pNL-RT_{WT}.
[e] N- and C-terminal regions of CL-6 contained T39A/M41L/K43E/D67N/V75M/V118I/I132V/L210W/T215Y and N348I/I393L in their coding regions, respectively (see also Fig. S1 in the supplemental material).
[f] No resistance-associated mutations were observed in either the N- or C-terminal region of CL-9 (also see Fig. S1 in the supplemental material).

showed resistance (Fig. 1; see also Fig. S1 in the supplemental material) to NVP and DLV. Interestingly, the C-terminal region also conferred resistance to AZT and ddI even in the absence of AZT resistance mutations that normally reside at the N-terminal region within amino acids 41 to 219. Recently, mutations in the connection subdomain, including G335D, N348I, and A360T, have been shown to confer AZT resistance (28). In these clinical isolates the C-terminal region contained four unique mutations in the connection subdomain: G335D, N348I, A360T, and I393L (see Fig. S1 in the supplemental material). G335D and A360T were continuously observed at every time point and are polymorphisms related to subtype D. Since these isolates showed no phenotypic resistance (Table 1 and Fig. 1B), it is unlikely that G335D and A360T are involved in the resistance, at least in subtype D. I393L was also continuously detected from time point 1 but disappeared after the treatment interruption at time point 9 (Fig. 1) while N348I appeared only from time points 4 to 6 and at point 8 under treatment.

To further clarify the effect of mutations at residues 348 and 393 on drug resistance, we generated the N348I and/or I393L mutations in the C-terminal region by site-directed mutagenesis on a pNL-RT_{WT} background. Consistent with the phenotypic experiments and the experiments with chimeric viruses, we found that the N348I substitution conferred resistance to AZT, ddI, NVP, and DLV. In contrast, we found that the I393L mutation caused no significant resistance by itself (Table 2). Furthermore, the combination of I393L with N348I did not show any significant increase in NVP resistance compared to N348I alone.

To address whether N348I further increases the level of AZT resistance in the presence of TAMs, we examined the effect of N348I on AZT susceptibility in the presence or absence of the classical AZT resistance mutations M41L/T215Y. M41L/T215Y or N348I showed only moderate resistance to AZT whereas a combination of M41L/T215Y and N348I further enhanced AZT resistance (Table 2). These data demonstrate that the N348I mutation is responsible for this cross-resistance to multiple members of the NRTI and NNRTI families and enhances AZT resistance induced by TAMs.

Viral replication kinetics. Since N348I and I393L immediately disappeared after cessation of HAART, we examined whether these mutations have an effect on viral replication kinetics using the p24 antigen production assay and a competitive HIV-1 replication assay (CHRA). In the p24 antigen production assay, acquisition of N348I drastically impaired replication in MT-2 and SupT1 cells (Fig. 2A and B). However, a moderately low reduction of replication kinetics was observed in PM1, H9 cells, and PHA-stimulated PBMCs (Fig. 2C, D, and E). HIV-1 carrying the mutation I393L (HIV-1_{I393L}) showed comparable replication kinetics in all cells tested. A combination of I393L with N348I showed no apparent change of replication kinetics in MT-2, SupT1 cells, and PHA-stimulated PBMCs (Fig. 2A, B, and E) and reduction in PM1 cells (Fig. 2C) compared to N348I alone. CHRA was performed for further comparison of replication kinetics in H9 cells. During 6 weeks in culture, we observed little difference in viral replication in H9 cells (Fig. 2F). A lack of an effect of I393L on the replication of N348I was confirmed by CHRA (Fig. 2G). These results indicate that N348I impairs viral replication in a cell-type-dependent manner and that I393L exerts little effect on viral replication of either the WT or N348I clones. Thus, I393L appears to be one of the specific polymorphisms for this isolate.

Insertion at 69 and N348I. At time point 8 we detected the transient presence of the fingers insertion mutation, a 2-amino-acid insertion at codon 69 in the presence of TAMs known to confer resistance to NRTIs by enhancing the excision reaction (3) (Fig. 1). Interestingly, at time point 8 WT N348 coexisted with resistant I348. To address whether these two MDR mutations were introduced onto the same RNA genome, we carried out clonal sequence analysis of PCR products. The results show that the fingers insertion and the N348I mutations were randomly introduced; seven, three, one, and six clones ($n = 17$) contained both mutations, the fingers insertion only, N348I only, and no mutation or insertion, respectively, in the background of TAMs (Table 3). In previous studies the fingers insertion complex emerged with the K70E mutation that was selected in vitro with adefovir (8) and β-2′,3′-didehydro-2′,3′-dideoxy-5-fluorocytidine (18), and it conferred low level resistance to TDF, ABC, and 3TC (39). The effect of K70E on resistance or enzymatic activity influenced by the fingers insertion remains to be elucidated. These results suggest that there is no correlation between the N348I and the

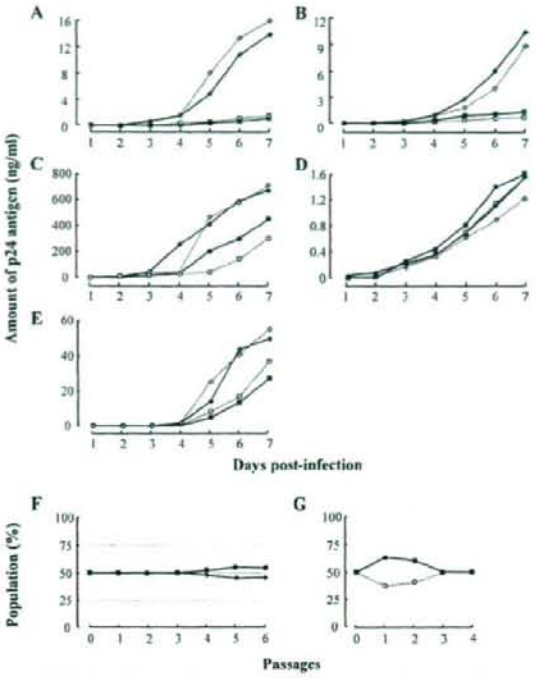


FIG. 2. Viral replication kinetics. Production of p24 antigen in culture supernatant was determined with a commercially available p24 antigen kit. Profiles of replication kinetics (p24 production) of HIV-1_{WT} (closed diamonds), HIV-1_{N348I} (closed squares), HIV-1_{I393L} (open diamonds) and HIV-$1_{N348I/I393L}$ (open squares) were determined with MT-2 (A), SupT1 (B), PM1 (C) and H9 cells (D) and PHA-stimulated PBMCs (E). Representative results from at least two (or three) independent single determinations of p24 production with newly titrated viruses are shown. A competitive HIV-1 replication assay was performed in H9 cells to compare the replication kinetics of HIV-1_{WT} (closed diamond) and HIV-1_{N348I} (closed squares) (F) and of HIV-1 N348I (closed squares) and HIV-$1_{N348I/I393L}$ (open square) (G).

finger insertion mutations. Because our studies show that N348I does not confer d4T resistance, we speculate that the fingers insertion mutation was introduced to overcome the drug pressure by d4T.

TABLE 3. Sequences of HIV-1 RT-coding region of clinical samples

No. of clones[a]	Resistance-associated and unique mutation at the indicated position									
	M41	D67	T69	K70	V75	V118	L210	T215	N348	I393
5	L	N			M	I	W	Y		L
3	L	T	SSG	E	M		W	Y	I	L
3	L	T	SSG	E	M	I	W	Y	I	L
2	L	T	SSG	E			W	Y		L
1	L	T	SSG	E			W	Y	I	L
1	L	T	SSG	E	M	I	W	Y		L
1	L				M					
1	L				M	I	W	Y	I	L

[a] The PCR product at time point 8 was subcloned and sequenced (n = 17).

TABLE 2. Drug susceptibilities of HIV-1 variants constructed by site-directed mutagenesis

Mutation[a]	EC_{50} (fold increase)[b]											
	NRTI								NNRTI			
	AZT	ddI	ddC	d4T	3TC	ABC	TDF	NVP	DLV	Loviride	EFV
WT	0.035 ± 0.01	2.3 ± 0.34	0.7 ± 0.13	3.6 ± 1.36	2.1 ± 0.2	3.4 ± 0.14	0.03 ± 0.01	0.04 ± 0.02	0.04 ± 0.01	1.4 ± 0.38	0.003 ± 0.0008
N348I	0.24 ± 0.04 (6.9)	12 ± 1.0 (5.2)	0.74 ± 0.58 (1.1)	2.9 ± 0.21 (0.8)	1.7 ± 0.36 (0.8)	3.4 ± 1.11 (1)	0.02 ± 0.01 (0.7)	1.07 ± 0.06 (27)	0.22 ± 0.04 (5.5)	2.4 ± 0.35 (1.7)	0.005 ± 0.0005 (1.7)
I393L	0.06 ± 0.01 (1.7)	2 ± 1.37 (0.9)	0.42 ± 0.23 (0.6)	1.8 ± 1.21 (0.5)	1.5 ± 0.74 (0.7)	2.4 ± 0.95 (0.7)	0.02 ± 0.01 (0.7)	0.05 ± 0.01 (1.3)	0.04 ± 0.01 (1.0)	2.2 ± 0.4 (1.6)	0.003 ± 0.001 (1)
N348I/I393L	0.23 ± 0.03 (6.6)	11.3 ± 1.53 (4.9)	0.49 ± 0.01 (0.7)	4.2 ± 1.12 (1.2)	1.7 ± 0.40 (0.8)	2.7 ± 0.26 (0.8)	0.02 ± 0.01 (0.7)	1.02 ± 0.51 (26)	0.28 ± 0.06 (7)	2.6 ± 0.42 (1.8)	0.005 ± 0.001 (1.7)
M41L/T215Y	0.28 ± 0.06 (8)	4.5 ± 1.55 (2)	ND	ND	ND	ND	ND	0.05 ± 0.01 (1.3)	0.04 ± 0.02 (1)	ND	0.002 ± 0.0004 (0.7)
M41L/T215Y/ N348I	1.37 ± 0.21 (39)	9.9 ± 0.99 (4.3)	ND	ND	1.4 ± 0.20 (0.7)	ND	ND	1.11 ± 0.69 (28)	0.15 ± 0.06 (3.8)	ND	0.002 ± 0.0004 (0.7)

[a] See Materials and Methods for the construction of clones.

[b] Data are means ± standard deviations from at least three independent experiments. The relative increase in the EC_{50} value compared with that in HIV-1_{WT} is given in parentheses. Boldface indicates an increase greater than threefold. ND, not determined.