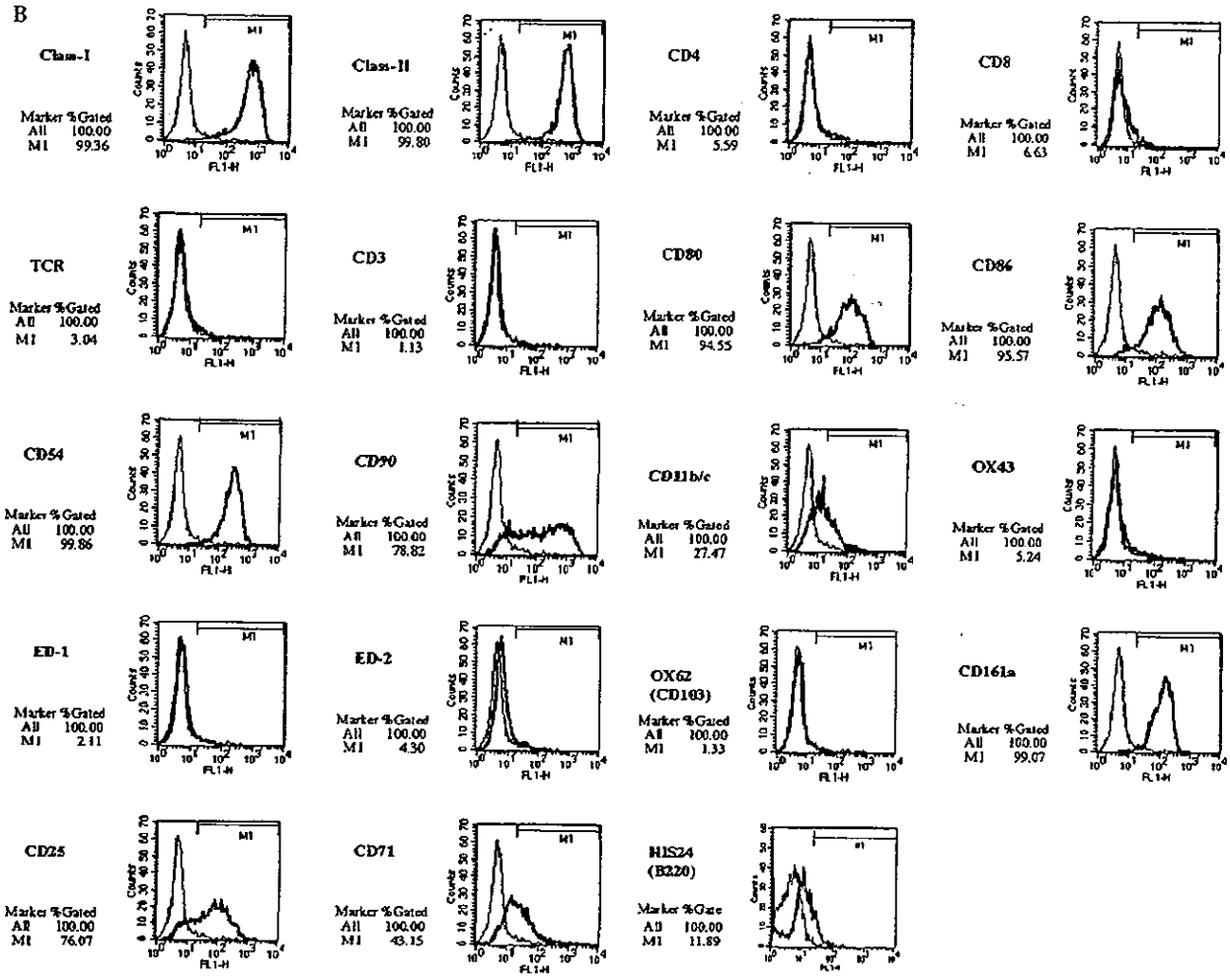

Fig. 2. (*continued*)

following experiments on in vitro as well as in vivo systems.

Fig. 3 illustrates typical figures of DC colonies under phase-contrast microscopic observation (A). Following autoMACS cell separation of CD161a$^+$ DC, described in Section 2, its cytospin preparation (B), phase contrast outlook of cell suspension (C), and May-Gruenwald and Giemsa staining (D) were shown.

3.4. Functional analysis of CD161+ DC in vitro

The most typical characteristic of DC as a specialized APC in vitro is that mature DC has profound capability to activate autologous or syngeneic T cells, in particular the CD4+ T cell subset, generating the so-called autologous or syngeneic MLC reaction. In allogeneic MLC it has become evident that both CD4+ and CD8+ T cells equally contribute to the responses, whereas in syngeneic MLC the contribution of CD8+ T cells is minimal or none. This is because fully matured DC expresses an enormous amount of class II on the cell surface, compared with any other known cells. In contrast to class II expression, the level of class I expression on fully matured DC appears not to be as high as class II, and skin Langerhans cells express nearly null class I in vivo [24]. Thus, in syngeneic MLC, CD4+ T cell–DC not CD8+ T cell–DC interaction [25] is unique and results in non-specific polyclonal activation of the CD4+ T subset in vitro. To examine the APC-activity of CD161a-selected DC in vitro, in particular for syngeneic MLC, we prepared two types of in vivo purified T cells from TDL. As described in Section 2, one source of TDL was prepared from LEW rat that had been lethally irradiated and subjected to the thoracic duct drainage, and further inoculated with normal LEW TDL. This population is known to contain more than 99% T cells [26]. Tentatively, this T cell population was labeled as LEW_{-LEW}. The other source of TDL was likewise labeled LEW_{-DA}. The latter LEW_{-DA} T cell preparation is important to evaluate whether and to what extent the LEW_{-DA} T cells respond to nominal antigens such as heterologous serum such as FBS in the presence of allogeneic APC. Due to the MHC restriction, LEW_{-DA} T should not respond to any peptides in allogeneic MHC such as RT1avl of DA. Thus, LEW

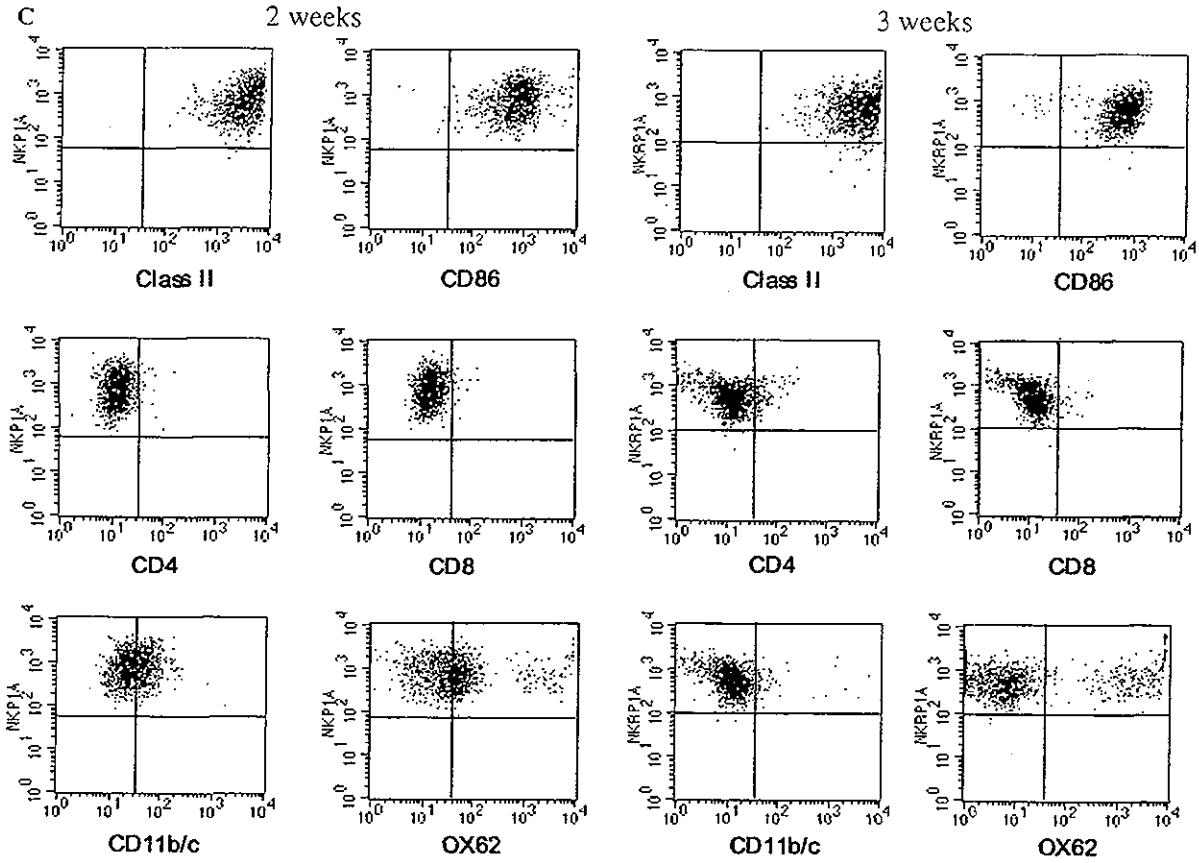

Fig. 2. (*continued*)

TDL was injected into the lethally irradiated DA rat that had been subjected to similar thoracic duct drainage.

Classical local GvH assay demonstrated that LEW_{-LEW} T cells were active to both allogeneic DA (RT1avl) in (DA × LEW) F1 and PVG (RT1c) in (LEW × PVG) F1, whereas LEW_{-DA} T cells were functionally inactive with respect to allogeneic MHC of DA, however, the same cells retained their reactivity to third-party allogeneic MHC of RT1c in (LEW × PVG) F1 (Table 1).

In Fig. 4A (2.5% rat serum) and Fig. 4B (10% FBS) demonstrate that in vivo purified LEW_{-LEW} T cells responded in syngeneic MLC [27] were observed regardless of serum sources (syngeneic or xenogeneic). Likewise both in vivo purified LEW_{-LEW} T cells and LEW_{-DA} T cells were equally active in syngeneic MLC (Fig. 4C). Unexpectedly, for allogeneic MLC, $CD161a^+$ DC from DA was found to be able to activate LEW_{-DA} T cells significantly in the absence of heterologous serum such as FBS, although it was far less strong and dose-dependent manner than that of syngeneic $CD161a^+$ LEW DC (Fig. 4D).

3.5. Functional analysis of $CD161a^+$ DC in vivo

Lastly, we examined the in vivo function of $CD161a^+$ DC as a specialized APC. Employing weak histocompatibility antigen difference, rat H-Y antigen, we immunized female rats with isogeneic male DC and subsequently challenged them by male isografts and compared with female rats immunized with fresh BMC. Based on our previous studies and for screening purposes, a single dose of assays was performed. As shown in Table 2, regardless of the cell administration route, as few as 1×10^5 $CD161a^+$ male LEW DC were found to sensitize female rats with male-specific antigen H-Y, hence, female LEW rats rejected male isografts. However, intravenous administration of $CD161a^+$ DC failed to have this effect in DA rats under the immunization protocols. It should be noted that regardless of the route (subcutaneous, intra-peritoneal or intravenous) or sources of cells (fresh BMC or DC described as above), PVG.1U (RT1u), which is known genetically to be non-responder, failed to sensitize with H-Y antigen.

4. Discussion

Several findings emerge from our study.

First, unlike mouse systems [8], rat DC progenitor in BMC did not fully respond to GM-CSF in terms of the growth of DC progenitor. Murine GM-CSF, both mouse and rat origin, do have a similar capacity to form granulocyte and Mϕ colonies in agar medium cultures

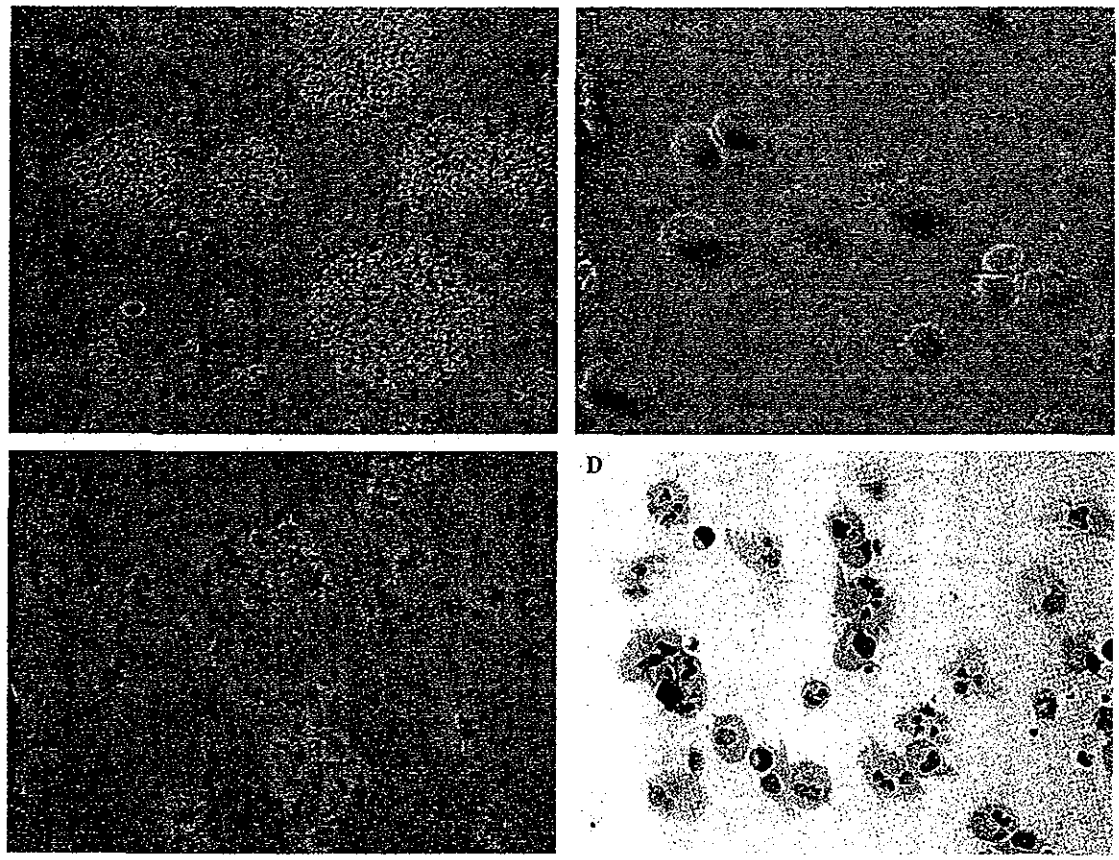

Fig. 3. Typical DC culture generated by Flt3/Flk2 ligand and IL-6 for eight weeks culturing was shown. When DC were generated spontaneously by Flt3/Flk2 ligand and IL-6, the most prominent picture was seen under the microscope. Large DC cell cluster or colony-like cell cluster appeared (A). With anti-CD161a (NKR-P1A), one of the C type lectin family, BMC-derived DC was positively separated by autoMACS systems as described in *Section 2*, and cytospin preparation was made following separation. Its fresh cytospin preparation with phase contrast image revealed the interference of phase contrast by magnetic bead attached on cell surface of DC. Hence its appearance looked as much smooth and as glossy (B). Phase contrast outlook of cell suspension for a short-term culture after the separation (C), and its staining by *May*–Grünwald and Giemsa was shown (D).

Table 1
Local GvH assay by recirculating T cells

Source of cell population	F1 hybrid	LN weight ± SE (mg)[a]	
		Injected side	Control
LEW_{-LEW} TDL[b]	DA × LEW	128 ± 21	5.1 ± 0.4
	LEW × PVG	99 ± 8	4.7 ± 0.2
LEW_{-DA} TDL	DA × LEW	7.0 ± 0.8	4.2 ± 0.1
	LEW × PVG	92 ± 6	4.8 ± 0.3

[a] Each estimates is the mean of four assay animals with 10×10^6.
[b] 10^7/0.1 ml of re-circulating T cells (>99% purity) were injected into foot pads of F1 recipients at day 7, popliteal lymph nodes were removed and weighed.

containing mouse and rat GM-CSF (data not shown). However, culturing rat BMC with a single supplement of GM-CSF failed to significantly increase DC yield. It appears that GM-CSF per se facilitates the differentiation of DC progeny rather than the growth of DC progenitors.

Second, combined cytokines of rat c-kit ligand and human IL-6 are able to support undefined BMC precursors by five hundred folds to one thousand folds within one month by in vitro culture [12], however, they fail to increase DC progeny of rat BMC. Indeed, the early expanded smooth-surfaced, round cells rapidly changed into large, irregular and spindled cells following a GM-CSF-containing medium, however, these cells were unable to differentiate into typical veiled-typed DC. Heretofore most undefined adherent cells were not fully characterized, how GM-CSF, TNF-α, and IL-4 influence the induction of DC is not known.

Third, combined Flt3/Flk2 ligand and IL-6 [21] appeared to support the growth of a low-density fraction BMC for at least the first four week's culture. It maintained the proliferation of round smooth-surfaced cells along with other adherent cells. Regarding the undefined adherent irregularly shaped cells, combined Flt3/Flk2 ligand and IL-6 culture systems resulted in far fewer adherent cells compared with those of GM-CSF supplemented cultures. At any time points of cultures, switching into the cocktails of cytokines such as IL-4 or TNF-α (data not shown), down-regulated the proliferation activity of smooth round-shaped cells. Thus,

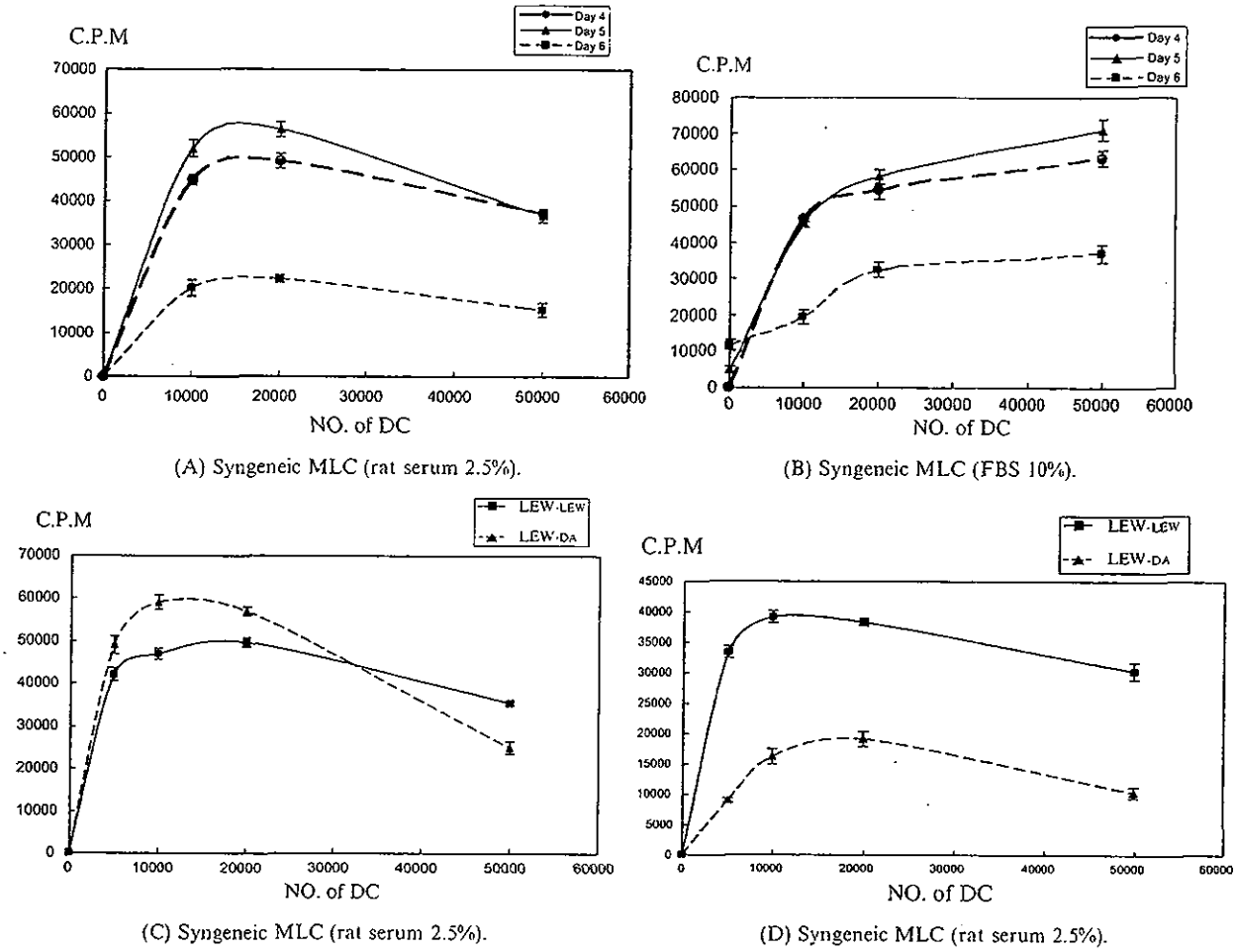

Fig. 4. (A) Highly purified LEW T cells (LEW_{-LEW}) (>99%) were prepared by collecting thoracic duct lymph filtration in vivo through lethally irradiated LEW rat that had been under the thoracic duct drainage and injected with 1.5×10^9 normal LEW TDL as described in Section 2. About 2×10^5 LEW_{-LEW} T cells were cultured with various numbers of $CD161a^+$ LEW DC generated with Flt3/Flk2 ligand and IL-6 from long-term bone marrow cell (BMC) cultures. During the syngeneic MLC cultures at day 4, day 5, and day 6, 0.5 μCi methyl-[^{3}H]thymidine of 25 μl was added to cell suspension in a 6 h pulse. In this culture 2.5% syngeneic LEW rat serum was employed. Optimal cell ratio and maximal activation occurred around 20T cell/1 DC and at day 4 to day 5, respectively. (B) Highly purified LEW T cells (LEW_{-LEW}) (>99%) were prepared by collecting thoracic duct lymph filtration in vivo through lethally irradiated LEW rat that had been under the thoracic duct drainage (LEW_{-LEW}) and injected with 1.5×10^9 normal LEW TDL as described in Section 2. About 2×10^5 LEW_{-LEW} T cells were cultured with various number of $CD161a^+$ LEW DC generated with Flt3/Flk2 ligand and IL-6 from long-term bone marrow cell cultures. During the syngeneic MLC cultures at day 4, 5, and 6, 0.5 μCi methyl-[^{3}H]thymidine of 25 μl was added to cell suspension in a 6 h pulse. In this culture 10% FBS was employed. In the presence of heterologous serum such as FBS, optimal cell ratio was not determined. However maximal activation likewise occurred at day 4 to 5. (C) In order to determine the relative difference between purified LEW T cells filtered through syngeneic LEW or allogeneic DA rat with syngeneic MLC reaction, reactivity was estimated at single day 4 that appears to be maximal [^{3}H]thymidine incorporation. Thus 2×10^5 LEW_{-LEW} and LEW_{-DA} T cells were likewise cultured with various numbers of $CD161a^+$ LEW DC generated with Flt3/Flk2 ligand and IL-6 from long-term bone marrow cell cultures. (D) In order to determine the functional difference between purified LEW T cells filtered through syngeneic LEW or allogeneic DA rat with allogeneic MLC reaction, reactivity was estimated at single day 5 that appears to be maximal [^{3}H]thymidine incorporation. Thus 2×10^5 LEW_{-LEW} and LEW_{-DA} T cells were likewise cultured with various number of $CD161a^+$ DA DC generated with Flt3/Flk2 ligand and IL-6 from long-term bone marrow cell cultures. Although it was weaker than LEW_{-LEW}, LEW_{-DA} T cells were significantly activated with a wide range of $CD161a^+$ allogeneic DA DC. This was sharp contrast to in vivo reactivity of LEW_{-DA} to DA alloantigen.

typical veiled-shaped, free-floating VC or DC appeared in substantially large numbers along with large irregularly shaped adherent cells that outnumbered the DC colony. A population of DC-committed progeny of BMC appeared to account for a small percentage of cells compared with those of Mϕ-committed progeny. It should be noted that additional cytokines such as GM-CSF, IL-4 and/or TNF-α rendered proliferating free round cells into irregularly shaped adherent cells. Differentiation of DC-committed progeny into fully mature DC, however, was found not to be totally dependent on these cytokines. Rather these cytokine combinations appear to facilitate the DC progeny to differentiate further into fully mature DC.

Regarding the phenotype of DC specific markers, no single-cell surface marker has been able to define DC

Table 2
Comparison of the effect of fresh BMC and DC prepared from male donor on sensitization of female recipients

Experimental animals	Source of cell used for sensitization	Source of cells	Graft survivals (days)[a]	Median of survival times (MST)
LEW ♂ → ♀ LEW	IV[b]	Fresh BMC	12, 12, 13, 15, 16, 17	15
	SC[b]		14, 14, 15, 17, 78	14
	IV	DC[c]	21, 27, 42, 58, > 100	42
	SC		10, 11, 12, 18, 23, 57	15
DA ♂ → ♀ DA	IV	Fresh BMC	68, 9× > 100	100
	SC		19, 21, 23, 28, 2× > 100	25.5
	IV	DC	23, 17× > 100	100
	SC		17, 18, 24, 33, 48, 3× > 100	40.5
PVG.RT1U ♂ → ♀ PVG.RT1U		Fresh BMC	ND[d]	
	IV	DC	4× > 100	100
	SC		8× > 100	100

[a] Following the immunization of female rats with male cells, one month later the recipient rats received male skin isografts as described in Section 2. The fate of the graft was followed.
[b] Fresh male BMC without culture were prepared as described in Section 2. Two doses of 20×10^6/0.1 ml of cells were injected either in the foot pads (subcutaneous, SC) or through the tail vien (IV).
[c] Male DC from the cultured BMC as described in Section 2 were prepared. Two doses of 0.1×10^6/0.1 ml of cells were injected either in the foot pads (SC) or through the tail vien (IV).
[d] ND: not determined.

specifically. Although in mouse DC, CD11c is widely used to define a subset of DC, equivalent rat CD11b/c (OX42) was expressed at an extremely low level or null on rat DC described here. Furthermore, classification of mouse *plasmacytoid* DC generated by Flt3 ligand is characterized by CD11c expression with B220 (CD45R), and proposed by others to distinguish the *plasmacytoid* or *lymphoid* DC from myeloid DC. The latter differentially expressed CD11b but not B220 [28]. With this respect, the rat DC generated in this study appears to belong to neither typical plasmacytoid nor lymphoid DC subsets.

As for the DC specific marker, CD103 (MRC-OX62) has been characterized by Brenan and Puklavec [16] and has been considered to be a specific marker for a subset of DC. Two subsets of rat DC have been reported; (i) $CD103^-$ phenotype consisting of DC in the epidermal Langerhans cells [16], and (ii) $CD103^+$ phenotype consisting of DC-like veiled cells in the thoracic duct lymph (TDL) [16,29,30]. Additionally, $CD161a^+$ (also defined as NKR-P1A [23,31]) was found to be positive for a subset of rat DC. It is tempting to define rat DC by two markers, CD103 and CD161a. By this criteria, DC driven by Flt3/Flk2 ligand and/or IL-6 appear to be defined $CD161a^+$ $CD103^-$. In this study, we confirmed and extended the study by Brissette-Storkus et al. [32]. These investigators also described a short-term rat BMC culture driven by *Flt3/Flk2 ligand* in FBS based culture medium resulting in $CD161a^+$ $CD103^-$ phenotype DC, although the expression level of CD161a appears to be far below that of our study. Nevertheless, the level of CD161a as well as class II expression on rat DC cultured in the present study gradually increased during a long-term culture. It appears that the level of CD161a is intimately related to the maturation processes of DC progeny. Whether CD161a expression of DC during the maturation processes is a general feature of the DC subset or only a unique characteristic of rat DC remains to be determined.

The view of that GM-CSF plays a primary role in growth and differentiation of DC precursor cells, was first suggested by Steinman and his colleagues in mouse systems [8,9,33]. However, based on our study, it is tempting to postulate that GM-CSF per se has a limited capacity to increase DC progenitors of BMC but instead acts as a differentiation factor in general. It should be noted that both the GM-CSF deficient mouse and the GM-CSF receptor deficient mouse contained a substantial number of DC, although the level of the cell number was significantly lower than both the normal mouse and the GM-CSF transgenic mouse [34]. In this regard, our preliminary and on-going studies (manuscript in preparation) demonstrate that our culture systems are likewise applied to generate *myeloid DC* from BMC, not only from the GM-CSF deficient mouse and GM-CSF receptor deficient mouse [35], but also in the human system. Thus, our culture systems demonstrate that Flt3/Flk2 ligand combined with IL-6 is able to replace the effect of GM-CSF on the outgrowth of DC precursors from the BMC culture. These results strongly suggest a common pathway, which is *GM-CSF independent myeloid DC* development [15,36,37]. Different subsets of DC-precursors might require different cytokines, depending on the cell-differentiation stages. Nevertheless, at least in the rat system, the absolute requirement of exogenous GM-CSF for BMC-derived *myeloid DC induction* appears to be less likely.

As for the function of *GM-CSF independent myeloid DC*, the APC function of CD161a^{+} DC generated by *GM-CSF independent* rat BMC culture driven by Flt3/Flk2 ligand and IL-6 was verified by in vitro syngeneic MLC as well as in vivo sensitization of minor histocompatibility antigen H-Y, which is considered to be a prototype of tumor immunology. Thus, the simple culture methods described here would facilitate the acquisition of a large number of highly uniform subsets of DC for cellular immunology research including transplantation biology as well as tumor immunology.

Heretofore, valuable information and knowledge have been accumulated on the prerequisite cytokines for DC development and classification of DC subsets. However, it remains to be determined whether and to what extent each cytokine, or cytokines in concert, play a crucial role in growth and/or differentiation of particular DC subsets from hematopoietic stem cells, and whether to what extent so-called DC specific markers are stably expressed during the their life span. Our simplified culture method described here may be applied to further investigation of the functional and developmental aspects of DC subset.

In summary, we have demonstrated that GM-CSF per se is not able to support a significant growth of DC progenitors in rat BMC, regardless of the sources (mouse, rat, human). Combined with Flt3/Flk2 ligand and IL-6, not with c-kit ligand and IL-6, undefined DC progenitors emerge, and these cytokines appear to be able to increase DC progeny from rat BMC. Thus, these expanded DC precursors are able to differentiate into fully mature DC in both phenotype and function as specialized APC. Functionally full-mature DC express at a high level of CD161a and hence the mature DC were highly purified by this single cell surface marker. With additional cytokines such as murine GM-CSF, TNF-α and IL-4, it appears that heterogeneity of phenotypic and functional DC [38–40] is generated. These additional cytokines in concert, not a single cytokine, might enable generating phenotypic and functional diversity of DC in vivo.

Acknowledgments

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Study of Antiretroviral Drug–Resistant HIV-1 Genotypes in Northern Thailand: Role of Mutagenically Separated Polymerase Chain Reaction as a Tool for Monitoring Zidovudine-Resistant HIV-1 in Resource-Limited Settings

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Summary: As the number of HIV-1–infected individuals receiving antiretroviral drugs has been rapidly increasing in developing countries, there is an urgent need for drug resistance genotype information of non-B subtype HIV-1 and for the establishment of a practical system of monitoring drug-resistant viruses. This study first sequenced the reverse transcriptase region of HIV-1 in 112 infected individuals who had been treated with zidovudine (AZT)/didanosine or AZT/zalcitabine as dual therapy at a government hospital in northern Thailand and then compared the above sequence method with mutagenically separated polymerase chain reaction (MS-PCR) for detecting M41L and K70R mutations. Concordant rates of detecting M41L and K70R mutations by the 2 methods were 96.9% (93/96) and 92.7% (89/96), respectively. The M41L and K70R MS-PCR could detect 86.4% of AZT-resistant strains with any resistance mutation, which was determined by the sequencing method. Then 292 drug-naive individuals were screened for the presence of drug-resistant HIV-1 by the MS-PCR assay and it was found that 2 individuals (0.7%) carried viruses with either the M41L or K70R mutation. It is feasible to test a large number of samples with MS-PCR, which is sensitive, cheap, and easy to perform and does not require sophisticated equipment. The M41L and K70R MS-PCR is potentially a useful tool to monitor the spread of AZT-resistant HIV-1 in resource-limited countries.

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HIV-1 has tremendous ability to mutate swiftly and to develop resistance to almost all clinically used antiretroviral drugs. Reduced sensitivity to nucleoside reverse transcriptase inhibitors (NRTIs), non-NRTI (NNRTIs), and protease inhibitors has been studied extensively and is linked to specific point mutations in either the reverse transcriptase or the protease gene.[1] Most current knowledge for interpreting these genotypic changes has been derived from studies on HIV-1 subtype B viruses. Worldwide, however, the majority of HIV-1–infected people live in developing countries and most of them are infected with non-B subtypes. Non-B subtypes differ from subtype B in *pol* gene by 10–15%.[2] We and other groups have published data showing some discrete differences in the patterns of drug resistance mutations between subtypes.[3–5] With a growing demand for access to antiretroviral therapy in resource-limited countries, the resistance patterns of non-B subtype viruses to antiretroviral drugs are becoming an important issue.

In Thailand, with a population of approximately 62 million, it was estimated that 695,000 people were living with HIV-1 infection. Of these, the majority are infected with CRF01_AE (previously known as subtype E), and 55,000 people had AIDS in 2000.[6] In the past, only a small minority of HIV-1–infected patients could afford antiretroviral drugs due to the high monthly price; thus most were either not treated or were treated with suboptimal antiretroviral regimens, mostly dual therapy.[7] When patients are treated suboptimally, HIV-1 acquires resistance to drugs more quickly.

Furthermore, the prevention of mother-to-child HIV-1 transmission (PMTCT) program, which uses a short-course zidovudine (AZT) regimen, was expanded first in northern Thailand in 1997 and later throughout the country.[8] This expanded program could also trigger the emergence of AZT drug resistance. Studies of HIV-1–infected individuals with primary HIV infection have shown that drug-resistant HIV strains can be transmitted from one adult to another and occasionally transmitted vertically from mother to child.[9–12] To control the spread of drug-resistant HIV-1, a monitoring system of antiretroviral drug-resistant HIV-1 in an epidemiologic scale is urgently needed.

The *pol* gene has been commonly sequenced for testing drug resistance in many HIV laboratories of developed countries. However, access to the sequencing test in developing countries is limited due to the relatively high costs of reagents and unavailability of expensive equipment such as an automated sequencer. Polymerase chain reaction (PCR)-based assays are an alternative method of detecting point mutations, having the advantage of increased sensitivity and low cost. Allele-specific primer extension assays have been applied to detect drug-resistant HIV-1; however, they have not been adequately specific for widespread application.[13,14] Conversely, mutagenically separated PCR (MS-PCR) is a PCR-based point mutation assay that overcomes this specificity limitation and has been successfully applied to detect drug-resistant HIV-1 of non-B subtypes.[15–17] Previous papers evaluated the performance of MS-PCR in developed countries, but it has not yet been used in resource-limited settings.

We conducted this study with the following objectives: to evaluate the performance of MS-PCR specific for M41L and K70R mutations in detecting AZT-resistant HIV-1 strains in Thailand; to apply the MS-PCR to the screening of AZT-resistant HIV-1 among drug-naive HIV-1–infected Thais; and to investigate the patterns and prevalence of drug-resistant genotypes among HIV-1–infected Thai individuals who had been treated with suboptimal antiretroviral regimens by sequencing the *pol* gene.

PATIENTS AND METHODS

Study Population

We used samples obtained from HIV-1–infected individuals who attended the Day Care Center clinic at the Lampang Hospital from July 6, 2000, to July 15, 2001 and gave a written informed consent. The Lampang HIV study was approved by the Thai government ethics committee. The Lampang Hospital is a government referral hospital with approximately 800 beds, situated in the center of Lampang province, which is 100 km south of Chiang Mai in northern Thailand. Plasma samples were collected from these individuals and stored at −80°C until their use. Viral load measurement was conducted using a commercial kit (Amplicor HIV-1 Monitor Test, version 1.5; Roche Diagnostics, Branchburg, NJ). CD cell count was measured by flow cytometory (FACScan; Beton-Dickinson, Franklin Lakes, NJ).

Direct Sequencing

After viral load measurement, the residual RNA w used for drug resistance genotyping by sequencing *pol* gene previously described.[17] Briefly, an 888-basepair (bp) rever transcriptase fragment (base number of nucleotide: 248 3372) was amplified by PCR after a reverse transcription (R reaction from the RNA by an RNA-PCR kit (AMV One St RNA PCR Kit; Takara, Osaka, Japan). Primary PCR produc were further amplified with a high-fidelity DNA polymera (KOD DNA polymerase; Toyobo, Osaka, Japan). Sequencir was performed using an autosequencer ABI-3100 (Applie Biosystems, Foster City, CA) with dye terminators (BigD Terminator Cycle Sequencing Ready Reaction Kit; Applie Biosystems). The sequence results were assembled an aligned on the reference HIV sequence of HIV-1 HXB2 (Ge Bank accession number M38432) by ABI Prism SeqScap Software (Applied Biosystems). They were submitted t Stanford HIV RT and Protease Sequence Databas (http://hivdb.stanford.edu/) for drug resistance genotyping.

M41L and K70R MS-PCR

On measuring the viral load, we used the residual RN for the MS-PCR experiments. The method of MS-PCR for de tecting M41L and K70R AZT resistance mutations i CRF01_AE has been described in a previously published pa per.[17] Briefly, the first-round RT-PCR was conducted to am plify a 370-bp RT region, which spans codon 41 and 70. Th second- and third-round PCR were conducted with 1 commo forward primer and 2 reverse primers, which are allele spe cific. The size of the wild-type specific primer was designe about 20 nucleotide bases longer than the mutant type-specifi primer so that the wild-type PCR product could be easily dif ferentiated from the mutant-type PCR product by electropho resis with a 3% agarose gel. We used clinical samples, o which drug-resistant genotypes were confirmed by the se quencing method used for positive controls, and included them for every experiment. When an MS-PCR result showed double bands, of which one was faint, we retested the sample.

Statistical Methods

We compared proportions by the χ^2 test. Means of continuous variables were compared by a nonparametric test, the Kruskal-Wallis one-way analysis of variance. The data management and statistical analysis were conducted using Epi Info version 6.04.

RESULTS

A total of 489 HIV-1–infected individuals attended the clinic during the observation period. History of antiretroviral

drug therapy was available from 487 infected individuals, in whom 336 were drug naive and 151 were drug experienced; 22 individuals were exposed to a single NRTI, 114 to two NRTIs, 14 to three or more antiretroviral drugs including protease inhibitors, and one did not have regimen information.

Patterns and Prevalence of Drug Resistance Mutations Among Suboptimally Treated Individuals

Out of the 114 individuals who had been exposed to 2 NRTIs, we first analyzed 112 individuals who had received AZT/didanosine (ddI) or AZT/zalcitabine (ddC) dual therapy for the sequencing of RT region; the other 2 individuals had received either d4T/ddC or AZT/lamivudine (3TC). A total of 108 individuals (96.4%) were infected via the heterosexual route. The median (range) duration of dual therapy was 621 (28–1600) days; 76 individuals were on therapy and 36 patients were off therapy at the time of sampling; 28 individuals were exposed to AZT/ddI, 76 to AZT/ddC, and 8 to both AZT/ddI and AZT/ddC. Sequence data were obtained from 99 individuals, of whom 57 (57.6%) had mutations that are known to be associated with AZT, ddI, or ddC resistance. We could not obtain sequence data from 13 individuals, mainly because of their low viral load; 10 had a viral load under the detectable level (<400 copies/mL). Assuming that the individuals without sequence data did not have any drug-resistant virus, the prevalence of drug-resistant viruses detected by the sequencing method was 57/112 (50.9%) among those who had received dual therapy. If confined to the 76 individuals on therapy, 48 (63.2%) had drug-resistant viruses. Figure 1 summarizes amino acid variations at the sites, which are known to be associated with AZT, ddI, or ddC resistance mutations in subtype B infection. The most common drug resistance mutations were D67N, followed by K70R and T215Y/F. Other mutations were also commonly seen at codon 41, 210, and 219. No mutation of Q151M was found. We also analyzed associations among these specific mutations. The presence of D67N was strongly associated with K70R and less significantly with M41L; 31 (86.1%) out of 36 individuals with D67N mutation had either a K70R or M41L mutation (Table 1). The presence of T215Y/F was strongly associated with M41L but not with K70R; 22 (71%) of 31 individuals with T215Y/F mutation had K70R or M41L mutation (Table 1).

TABLE 1. Associations of D67N and T215Y/F With M41L and K70R Mutations

Codon 67 Mutation						
	M41L*		K70R†		M41L or K70R*	
	Wild	Mutant	Wild	Mutant	Wild	Mutant
Codon 67						
D	57	6	55	8	49	14
N	26	10	12	24	5	31

*$P < 0.0001$, †$P = 0.017$.

Codon 215 Mutation						
	M41L*		K70R		M41L or K70R†	
	Wild	Mutant	Wild	Mutant	Wild	Mutant
Codon 215						
T	66	2	46	22	45	23
Y/F	17	14	21	10	9	22

*$P < 0.0001$, †$P = 0.0005$.

Drug Resistance Mutations in Relation to the Duration of Antiretroviral Therapy

The prevalence of drug resistance mutations correlated with the duration of dual therapy among 76 individuals on therapy. Among those with the duration of therapy for <180 days, 180–365 days, and >365 days, drug-resistant viruses were found to be predominant in 3 (30%), 8 (62%), and 33 (69%) individuals, respectively, with the median numbers of drug resistance mutations of 0, 2, and 2, respectively (Fig. 2). The number of drug resistance mutations was significantly associated with the median level of viral load: the median (interquartile range, IQR) viral load of individuals with no mutation, with 1–4 mutations, and with ≧5 mutations was 7412 (<400–62,432); 37,871 (7866–105,105); and 156,989 (32,682–184,767) copies/mL, respectively ($P = 0.018$ by Kruskal Wallis one-way analysis of variance).

Codon	41	44	65	67	69	70	74	108	118	151	184	210	215	219
Consensus B	M	F	K	D	T	K	L	V	V	Q	M	L	T	K
N=99	L(12) V(1) I(2)	D(3)	V(1)	N(33) E(2) G(2)	D(1) N(2) S(1) S_SG(1)	R(29)	V(1)	-	I(6)	-	-	W(13) F(2) M(1)	Y/ F(28) I(10) S(2) N(1)	Q/ E(20) N(1)

FIGURE 1. Patterns of AZT, ddI, or ddC resistance mutations. This figure shows amino acid variation at known AZT, ddI, or ddC resistance mutation sites. The frequency of each substitution is shown in parentheses. Reported drug resistance–associated mutations are shown in bold.

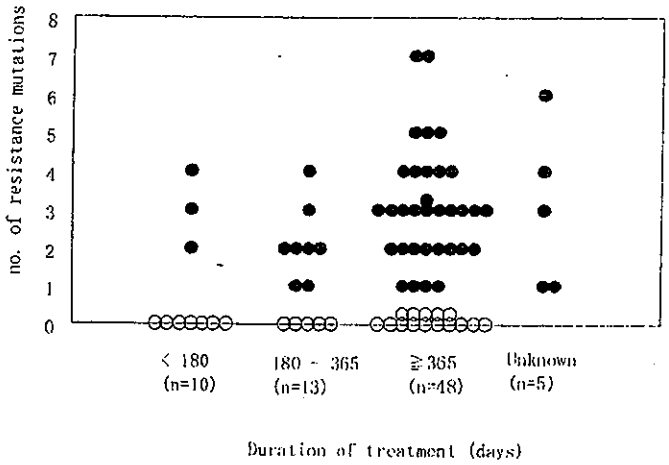

FIGURE 2. Prevalence of AZT, ddI, or ddC resistance mutations in relation to the duration of the dual therapy. Open circles indicate samples without resistance mutations.

Drug Resistance Mutations After Stopping Antiretroviral Drug Therapy

The interval between stopping dual therapy and the time of sampling was also associated with the presence of drug resistance mutations. Twenty-six individuals who had been substantially exposed to dual therapy for >180 days were off therapy at the time of sampling. Seven individuals (27%) were off therapy for >180 days and 19 (73%) were off therapy for <180 days. Drug-resistant viruses were detected in only 1 individual (14%) in the former group but 7 individuals (37%) in the latter group.

Evaluation of M41L and K70R MS-PCR in Detecting AZT-Resistant Strains

We then compared direct sequence methods with M41L and K70R MS-PCR in 96 antiretroviral drug–experienced individuals for whom both sequence and MS-PCR results were available (Table 2). Overall concordant rate for codon 41 was 96.9% (93/96) where M41I was regarded as a mutant type and concordant rate for codon 70 was 92.7% (89/96). Discordant results were seen mainly in the samples that were determined as mutant type by the MS-PCR and as wild type by the sequencing method.

To study the sensitivity of M41L and K70R MS-PCR as a screening strategy in detecting AZT-resistant strains, we defined the AZT-resistant strains as viruses with at least one AZT resistance mutation, which was detected by the sequencing method. Out of the 96 plasma samples that were tested by both the sequencing and the MS-PCR methods, 52 samples had no AZT resistance mutation and 44 samples had at least one AZT resistance mutation and were regarded as containing AZT-resistant viruses. Of the 44 samples with AZT-resistant viruses, the M41L and K70R MS-PCR detected either M41L or K70R mutation in 38 samples, resulting in the sensitivity of the M41L and K70R MS-PCR in detecting the AZT-resistant viruses at 86.4%. The number of AZT resistance mutations related to the detection rate of AZT resistance mutations by the MS-PCR (Table 3). When the viruses had multiple mutations, the sensitivity of the M41L and K70R MS-PCR was considerably higher. Of 39 samples containing HIV-1 with more than one AZT resistance mutation, 37 samples (94.5%) were diagnosed as having resistant viruses by the M41L and K70R MS-PCR.

TABLE 2. Comparison Between MS-PCR and Sequencing Results

Codon 41 Mutations			
	Sequencing Results		
	M	L	I
MS-PCR results			
Wild	84	1*	0
Mutant	2	7	2

*Sequence result of this patient showed a mixed type of M and L; it turned out to be mutant type when MS-PCR experiment was repeated.

Codon 70 Mutations		
	Sequencing Results	
	K	R
MS-PCR results		
Wild	65	1
Mutant	6	24

Screening AZT-Resistant Viruses Among Antiretroviral Drug–Naive Individuals in Northern Thailand

We applied the M41L and K70R MS-PCR to the screening of 292 antiretroviral drug–naive HIV-1–infected individu-

TABLE 3. The Sensitivity of M41L and K70R MS-PCR in Detecting AZT Resistance Mutations

AZT Resistance Mutations, n	Total	M41L and K70R MS-PCR Results		
		Wild	Mutant	Sensitivity
0	52	52	0	—
1	5	4	1	20%
2	14	1	13	92.9%
3	11	1	10	90.9%
≧4	14	0	14	100%

als attending the Lampang Hospital for the existence of AZT drug-resistant viruses. There were 271 individuals (92.8%) who were known to be infected with HIV-1 via the heterosexual route. We found 2 patients (0.7%) who carried mutant viruses: one had M41L and the other had K70R mutation. Later it was noted that these 2 patients, as well as their spouses, had never received any antiretroviral drugs but both had participated in clinical trials of herbal medicine in the past.

DISCUSSION

Our observation showed that AZT, ddI, or ddC resistance mutations were found in >50% of individuals who had received dual therapy. The prevalence of drug-resistant viruses was higher among individuals who had received the drugs for a longer period, as previously reported.[18,19] We attribute the high prevalence of resistant viruses to the fact that the dual therapy was suboptimal. Clinicians working in government hospitals, however, did not have other options because the more efficient antiretroviral therapy such as triple or quadruple therapy was not affordable for most patients when this study was conducted.[7] Recently, access to multiple antiretroviral drugs has been dramatically improved, because the Government Pharmaceutical Organization (GPO) started the production of generic antiretroviral drugs known as "GPOvir," which is a combined tablet of stavudine, lamivudine, and nevirapine. We nevertheless anticipate that individuals who had already had viruses resistant to NRTI dual therapy may not gain as much benefit from the generic medicine as antiretroviral drug–naive individuals do.

The most common mutations observed in this study were D67N, K70R, and T215Y/F, and we found few mutations at codons 65, 74, 108, 151, and 184. Such patterns of NRTI resistance mutations are similar to the patterns in CRF01_AE infection as well as in subtype B infections that have been reported in our previous report.[4] M184V mutation was often found in our previous study but not in the current study. We think that this difference reflects on the rare use of 3TC in Thailand when this study was conducted. Our current study, though a cross-sectional observation, showed several associations among resistance mutations such as D67N and M41L or K70R, T215Y/F and M41L in Thai strains as known in subtype B infection.[20,21]

We found a high concordance rate of MS-PCR with the sequencing method in detecting M41L and K70R point mutations. The finding is compatible with previous papers.[16,17] Discordant results between the MS-PCR and sequencing method were seen in some samples, most of which showed mutant type by the MS-PCR but wild type by the sequencing method. We think that such discordances are due to the greater sensitivity of MS-PCR for detecting a minor virus population than the sequencing method. However, a high sensitivity and specificity of detecting 2 particular point mutations do not specifically justify the application of M41L and K70R MS-PCR for the screening of AZT-resistant viruses in the field. D67N and T215Y/F mutations are very common but it is technically difficult to establish MS-PCR specific for these mutations due to a higher degree of polymorphism around the mutation sites. Our data showed that these mutations were frequently accompanied by M41L and/or K70R as previously reported in subtype B.[22] Furthermore, we evaluated how efficiently the M41L and K70R MS-PCR could detect AZT-resistant viruses that were detected by the sequencing. The overall sensitivity was reasonably high particularly among the viruses with multiple drug resistance mutations.

This is the first report that addressed the transmission of drug-resistant HIV-1 using a large number of samples in Thailand. We found that the prevalence of HIV-1 strains with either M41L or K70R mutation was as low as 0.7% among our drug-naive population. Considering that the overall sensitivity of the MS-PCR for detecting HIV-1 with any AZT resistance mutation was 86.4%, the prevalence of AZT-resistant HIV-1 was estimated to be 0.8%, which is still very low. There is still the concern that the low prevalence of resistant virus could be a consequence of the fact that the resistance to AZT in the drug-naive population was often associated with mutations at codon 60 or 215. To exclude this possibility, we further tested 60 samples, which were randomly selected from the drug-naive samples and confirmed that none had drug resistance mutations at these sites. The majority (127/292) of drug-naive individuals (43.5%) were initially diagnosed as HIV infected in 1997 or before, when the PMTCT program started in the region, and many were likely to have been infected several years prior to their first diagnosis of HIV infection. Thus, our result may not show an effect, which could have been triggered by the PMTCT program. A report from the United Kingdom suggests that transmission of drug-resistant HIV-1 is increasing.[23] We believe that our report is important in providing the baseline information on AZT-resistant HIV-1.

There has not been a consensus on the strategy of monitoring the transmission of drug-resistant HIV-1 in developing countries. Detecting individuals with primary viremia is ideal but not practical. In our study, we surveyed a drug-naive population for the presence of drug-resistant viruses. One concern with this approach is that drug-resistant viruses, which are generally less fit, might have been overwhelmed by the wild-type viruses in the absence of antiretroviral drug pressure because drug-resistant viruses among drug-treated individuals disappear following the interruption of antiretroviral therapy.[24] However, a recently published paper showed 2 cases of transmission of drug-resistant HIV-1 in which the resistant genotypes remained as a dominant population for a prolonged period in the absence of antiretroviral therapy.[25] Another way to monitor the spread of antiretroviral drug–resistant viruses is to screen infected individuals shortly after they receive antiretroviral therapy, which selects a minor population of insidious

resistant viruses, before de novo resistance mutations occur. Further studies are needed.

This study demonstrates that it is feasible to apply MS-PCR techniques for screening a large number of field samples for the presence of AZT-resistant viruses in Thailand. Taking into account the enormous benefits of MS-PCR such as much lower cost, ease of use, no requirement of automated sequencers, and higher sensitivity of detecting a minor virus population, we think that the M41L and K70R MS-PCR is a useful technique for the screening of AZT-resistant HIV-1 in epidemiologic surveys in developing countries. Recently, GPOvir has become widely available in Thailand. As the patterns of drug-resistant mutations against 3TC and nevirapine are relatively simple, we propose that MS-PCR technique should be considered for monitoring viruses resistant to this combination of antiretroviral drugs.

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Gag Non-Cleavage Site Mutations Contribute to Full Recovery of Viral Fitness in Protease Inhibitor-Resistant Human Immunodeficiency Virus Type 1

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It is well documented that human immunodeficiency virus type 1 (HIV-1) Gag cleavage site mutations (CSMs) emerge in conjunction with various HIV-1 mutations for protease inhibitor (PI) resistance and improve viral replication capacity, which is reduced by acquisition of the resistance. However, CSMs are not the only mutations that emerge in Gag during treatment; many mutations other than CSMs (non-CSMs) have been found to accumulate in the Gag region. In the present study we demonstrate the important role of Gag non-CSMs with regard to viral fitness recovery. We selected three Gag-protease sequences with different PI resistance-associated mutations and CSMs from patients with antiretroviral treatment failure. To clarify the significance of CSMs and non-CSMs, four types of recombinant viruses with different patterns in each sequence were constructed. These were the GP type (patient-derived Gag and protease), the P type (HXB2 Gag and patient-derived protease), the GP^{-c} type (CSMs removed from the GP type), and the P^{+c} type (CSMs in the HXB2 Gag frame and patient-derived protease). By comparison of these four types of recombinant viruses in each patient-derived Gag-protease sequence, we found that non-CSMs, which had no systematic pattern, make a significant contribution to viral fitness recovery. Our findings demonstrate a delicate interaction between the in vivo evolution of Gag and protease to evade drug selective pressure and the importance of Gag in evaluating drug-resistant viruses.

Human immunodeficiency virus type 1 (HIV-1) protease (PR) is the crucial enzyme for HIV-1 replication (23, 26). The enzyme cleaves the Gag precursor protein $p55^{gag}$ into six functional proteins (30, 33), which form the inner structure of the HIV-1 virion (19, 20). The ordered cleavage of the Gag p55 precursor protein by viral PR, the step which is required for viral maturation, is essential for the acquisition of infectivity of HIV-1 (27, 34, 39, 40, 42). Indeed, viral infectivity was lost by replacing the enzyme catalytic locus consisting of an aspartic acid at position 25 with asparagine (26). Hence, PR has been the major target of antiretroviral treatment (1, 11), and regimens with PR inhibitors (PIs) have been successful in suppressing viral replication to virtually undetectable levels in vivo (21). Still, a considerable number of patients were found to be unresponsive to such treatment because of the emergence of drug-resistant viruses (6, 16, 32, 37). Thus, more attention has been given to drug-resistant virus pathogenesis as the major impediment to successful antiretroviral therapy (ART). Recent studies have shown that, in general, acquisition of drug resistance may reduce virus replication capacity, i.e., viral fitness (13, 29, 43). This phenomenon seems to be evident, especially with regard to PI resistance-associated mutations (3, 7, 9). Many of the major PI resistance-associated mutations are located within the active site of the PR and significantly reduce PR activity (4, 9, 22, 38). This impact on fitness reduction on treatment outcome is noteworthy, as a previous study has suggested that drug-resistant viruses might be less pathogenic than wild-type viruses (12).

However, drug-resistant viruses have the potential to undergo further evolution to recover impaired PR activity. The continuation of drug selective pressure by ART may lead viruses to acquire additional mutations, which allows the viruses to recover their viral fitness (4, 22, 38). These compensatory mutations can appear not only in the PR itself but also in the natural substrate Gag region of the virus (10, 14, 17, 24, 44). Mutations in Gag cleavage sites are the mutations that can improve the replication capacity of PI-resistant viruses (31). Cleavage site mutations (CSMs) were most commonly found in the p2/p7, p7/p1, and p1/p6 cleavage sites (8). Several studies confirmed that p7/p1 (A431V) and p1/p6 (L449F) CSMs were important for viral fitness recovery in PI-resistant HIV-1 (2, 5, 28, 29, 36), and the importance of CSM is now widely accepted. When analyzing clinical samples, however, in addition to CSMs, many other mutations outside the cleavage sites, i.e., non-CSMs may also be found. The importance of non-CSMs in PI-resistant HIV-1 in vitro has been reported previously (18). However, the significance in the viral fitness recovery process in clinical isolates has heretofore not been clarified. Therefore, in this study we attempted to clarify the relevance of non-CSMs in clinically derived *gag-pol* sequences covering the p24 to p6 region. We found that non-CSMs also play a role as important as that of CSMs in the recovery of fitness of PI-resistant viruses.

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MATERIALS AND METHODS

Construction of PI-resistant molecular clones with combinations of p7/p1 and p1/p6 CSMs. To confirm the impact of p7/p1 (A431V) and p1/p6 (L449F) Gag CSMs on viral fitness in PI-resistant viruses, wild-type PR and two PI-resistant

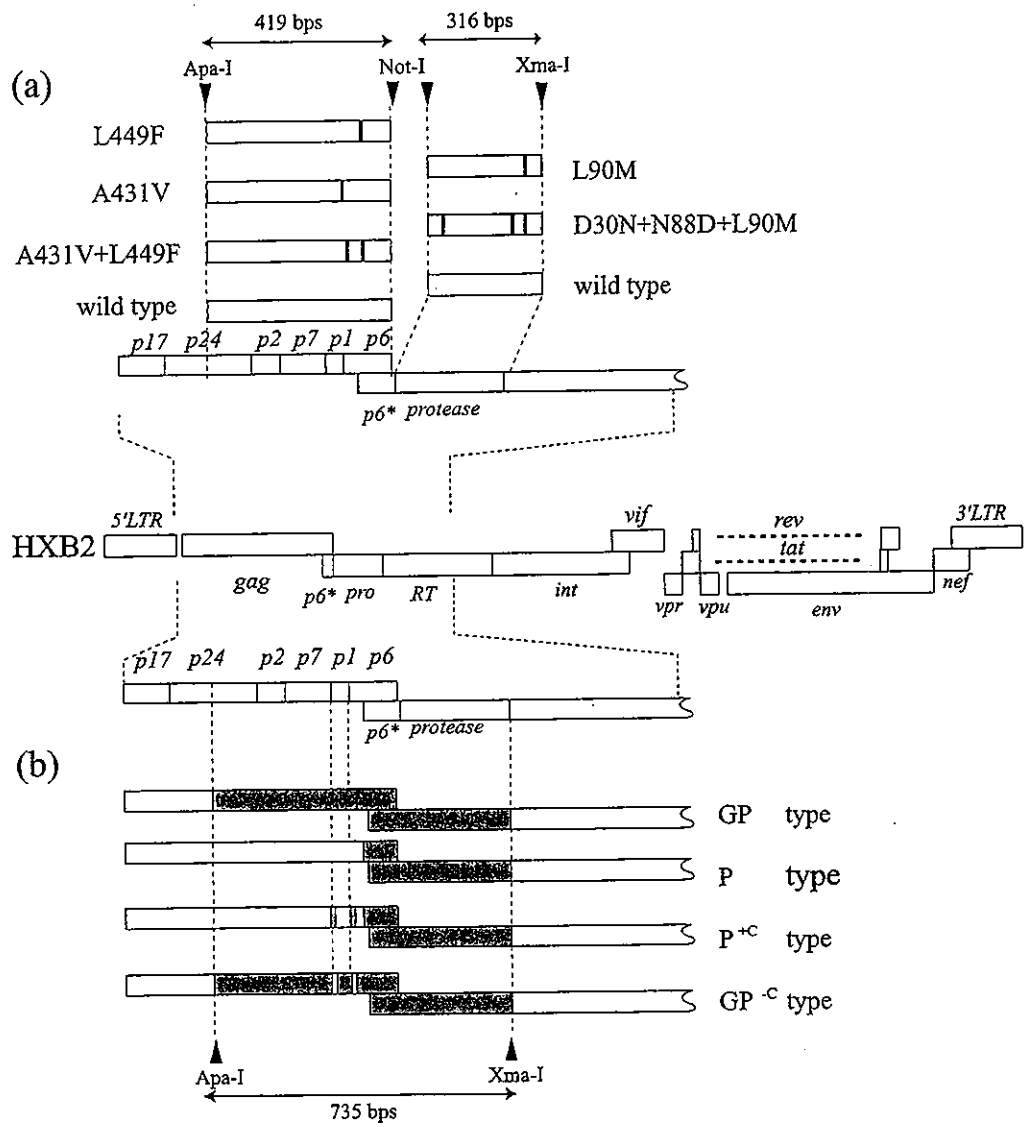

FIG. 1. Construction of recombinant viruses. (a) Preparation of viruses with artificially induced PI resistance with or without CSMs. Twelve clones with artificially induced PI resistance were constructed in the following manner: four *Apa*I to *Not*I *gag* fragments with different CSM patterns, and three *Not*I to *Xma*I protease fragments with three different PI resistance-associated mutation patterns, were prepared; by using these Gag and PR fragments, 12 different Gag-PR combinations were made and inserted into an HXB2 virus expression vector. (b) Construction of recombinant virus with a patient-derived Gag-PR fragment. Four different types of recombinants were constructed according to the pattern of the insert: (i) the GP type, (ii) the P type, (iii) the GP^{-c} type, and (iv) the P^{+c} type. LTR, long terminal repeat.

mutation patterns, (i) D30N, N88D, and L90M and (ii) L90M, were selected. With regard to the Gag region, wild-type Gag and three different CSM combinations, (i) A431V, (ii) L449F, and (iii) A431V and L449F, were selected. Recombinant viruses with these mutation patterns were constructed on a strain HXB2 backbone, as described previously (41). Preceding mutagenesis and construction of the recombinant clones, 448-bp *gag* regions (1849 to 2296) with *Apa*I (position 1856) and *Not*I (position 2275) restrictions sites were cloned into a pT7 cloning vector and used as the template in the subsequent site-directed mutagenesis to introduce CSMs. Primer pair DR431vF (5′ TTG TAC TGA AAG ACA GGT TAA TTT TTT AGG GAA GG) and DR431vR (5′ CCT TCC CTA AAA AAT TAA CCT GTC TTT CAG TAC AA) was used to introduce the A431V mutation. Primer pair DR449fF (5′ GGA GGC CAG GGA ATT TTT TTC AGA GCA GAC CAG AA) and DR449fR (5′ TTC TGG TCT GCT CTG AAA AAA ATT CCC TGG CCT CC) was used to introduce the L449F mutation. Mutagenesis was performed with KOD DNA polymerase (Toyobo Co. Ltd., Osaka, Japan) with PCR conditions of 94°C for 20 s, 52°C for 10 s, and 72°C for 90 s for 25 cycles. The results of the mutagenesis were confirmed by sequencing with an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, Calif.). Finally, a 420-bp *gag Apa*I-*Not*I fragment (positions 1856 to 2275) with A431V or L449F, or both, was cloned into the corresponding *gag* portion of each PI-resistant clone (Fig. 1a).

Patient sample selection and construction of recombinant viruses with patient-derived Gag and PR sequences. To investigate the significance of CSMs and non-CSMs in patient samples, we constructed the recombinant viruses with patient-derived Gag and PR sequences. For comparison with the results of earlier molecular cloning studies, we surveyed patient samples for CSMs in Gag (A431V or L449F, or both) and a D30N or an L90M mutation (or both) in the PR region. As D30N and L90M were targeted for the survey, samples from patients who failed a nelfinavir-containing regimen were focused on when samples sent to the AIDS Research Center of the National Institute of Infectious Diseases of Japan for routine drug resistance testing were analyzed.

Four different types of recombinant viruses were constructed for each patient Gag-PR fragment. These were (i) the GP type, which contained patient-derived Gag and PR sequences; (ii) the P type, which contained a patient-derived PR

sequence and the Gag region of wild-type strain HXB2; (iii) the GP^{-c} type, which contained a patient-derived Gag-PR sequence and wild-type CSMs, which were inserted by site-directed mutagenesis (therefore, there are only non-CSMs in the Gag region of this clone); and (iv) the P^{+c} type, which contained a patient-derived PR sequence and HXB2 wild-type Gag with the CSMs found in the corresponding GP clone (Fig. 1b).

The four types of recombinant viruses were constructed as follows. HIV-1 RNA was extracted from 200 μl of patient plasma by using a commercially available extraction kit (Roche Molecular Biochemicals, Mannhein, Germany). The extracted RNA was reverse transcribed with avian myeloblastosis virus reverse transcriptase (RT; TaKaRa, Otsu, Japan), and a 757-bp fragment (from positions 1849 to 2605), including p24, p2, p7, p1, p6, and PR, was amplified by nested PCR with Pyrobest DNA polymerase (TaKaRa). For this amplification, primers DRGAG5 (5′ TGT TAA AAG AGA CCA TCA ATG AGG AAG CTG) and DRPRO2 (5′ ATT TTC AGG CCC ATT TTT TGA) were used for the outer PCR and primers DRGAG7 (5′ ATA AAG CAA GAG TTT TGG CGG AAG CGA TGA GC) and DRPRO4 (5′ CTG GCT TTA ATT TTA CTG GTA) were used for the inner PCR. Subsequently, the 757-bp fragment that was amplified was cloned into the pCR-Blunt II-TOPO vector (Invitrogen, Carlsbad, Calif.). The sequences of the cloned fragments were verified by sequencing, and three fragments were selected for the study.

To insert the selected *gag-pol* fragment into the modified HXB2 vector (HXB2cv), unique *Apa*I and *Xma*I restriction sites were introduced at the 5′ ends and the 3′ ends of the fragments, respectively, by using forward primer DRGagPol-1 (5′ GGA GGG CCC GGC CAT AAA GCA AGA TTT TG) and reverse primer DRGagPol- 2 (5′ GGG CCA TCC ATC CCG GGC TTT AAT TTT ACT GG). Forward primer DRPolF1 (5′ AAC TCT TTG GCA GCG GCC GCT CGT CAC AAT AAA GAT) and reverse primer DRGagPol-2 were used to introduce the restriction sites *Not*I at the 5′ end and *Xma*I at the 3′ end of the 346-bp PR fragment (2260 to 2605). By this process, *Not*I and *Xma*I restriction sites were added to the 5′ and 3′ ends of the PR fragment, respectively. The GP-type and P-type recombinant viruses were constructed by using the introduced restriction sites.

To construct GP^{-c} clones, CSMs were removed and replaced with the wild-type HXB2 amino acid pattern by site-directed mutagenesis. Primer pair DR431aF-1 (5′ GAT TGT ACT GAA AGA CAG GCT AAT TTT TTA GGG AAG GTC) and DR431aR-1 (5′ GAC CTT CCC TAA AAA ATT AGC CTG TCT TTC AGT ACA ATC) and clone 1-1 were used to revert the A431V nucleotide to the alanine found in the wild type. Since the primer target site of clone 2-2 had a different sequence, a different primer pair, DR431aF-2 (5′ GAT TGT ACT GAG AGA CAG GCT AAT TTT TTA GGG AAG GTC) and DR431aR-2 (5′ GAC CTT CCC TAA AAA ATT AGC CTG TCT CTC AGT ACA ATC), was used. Primer pair DR449LF (5′ GGA GGC CAG GGA ATT TTC CTC AGA GCA GAC CAG AAC C) and DR449LR (5′ GGT TCT GGT CTG CTC TGA GGA AAA TTC CCT GGC CTC C) and clones 1-1 and 1-2 were used to revert the L449F nucleotide to the leucine found in the wild type. For the preparation of P^{+c} clones, 316-bp *Not*I-*Xma*I PR fragments (positions 2275 to 2590) were inserted into the HXB2 proviral DNA with the corresponding CSMs, which were prepared in the molecular clone experiments that had been carried out earlier as described above.

Evaluation of recombinant virus clone fitness in MT-2 cells. To evaluate the growth kinetics of the recombinant viruses, 10 μg of the constructed recombinant proviral DNA was introduced into 5×10^6 MT-2 cells by electroporation (250 V, 250 μF) with a Gene Pulser II apparatus (Bio-Rad, Hercules, Calif.). After the transfection, the cells were cultured in 10 ml of RPMI 1640 medium (Sigma Chemical, Taufkirchen, Germany) with 10% fetal calf serum (HyClone Laboratories, Inc., Logan, Utah). Half of the culture medium was harvested and replaced with fresh medium every 2 to 3 days, and the culture was maintained for up to 28 days posttransfection. The growth kinetics of the viruses were monitored through observation of the RT activities of the harvested supernatants. The RT activities were measured by a standard RT assay protocol described previously (15). For each recombinant virus, the supernatant with the highest RT activity was selected as the virus stock for the subsequent infectivity studies. The 50% tissue culture infective doses of the virus stock were determined by the limiting dilution method with the supernatant and were calculated by the Reed-Muench method. The growth kinetics of each recombinant virus were reproduced twice by use of independent transfections.

Evaluation of recombinant virus clone fitness by one-to-one competition cultures. To confirm the levels of replication competencies of the reconstructed clones, one-to-one growth competition cultures were performed. Each test virus (50% tissue culture infective dose, 100) was independently used to infect 2×10^6 MT-2 cells by incubation for 2 h at 37°C in individual test tubes. After the incubation, the infected MT-2 cells were washed once with fresh complete medium to remove excess virus inocula, and then the two tubes were combined in a T12.5 culture flask. Each competition culture was maintained for up to 28 days postinfection. Half of the culture medium was replaced with fresh complete medium every 2 to 3 days, and 10^5 MT-2 cells were added every 5 to 7 days. The proportion of the two test viruses in each competition culture was evaluated by cloning the *gag*-protease fragments of the viruses in the culture supernatant on days 0, 7, 14, 21, and 28 postinfection. Viral RNA was extracted from 200 μl of the culture supernatant, and a 757-bp *gag*-PR fragment was amplified by the same protocol described above to amplify the patient viruses. The amplicons were cloned into the pCR-Blunt II-TOPO vector and transformed into TOP10 competent cells by a heat shock procedure. Twenty clones were selected and sequenced at each time point, and the proportions of the two viruses were assessed.

RESULTS

Impact of p7/p1 CSM A431V and p1/p6 CSM L449F on replication of PI-resistant clones. To confirm the significance of the p7/p1 and p1/p6 CSMs, the growth kinetics of 12 clones with artificially induced PI resistance in independent cultures were monitored by measuring the RT activities in the culture supernatants. The results are shown in Fig. 2a to c.

For the wild-type PR, the virus with wild-type Gag and the virus with the L449F CSM grew at the same rate, and the peak RT activities of these two clones were observed at 11 days posttransfection. It appears that the L449F mutation had little or no effect on virus replication when the wild-type PR was present. A delay in viral growth was observed in the viruses with the A431V mutation and the A431V and L449F mutations. The time of peak RT activity of the virus with the A431V mutation and the virus with the A431V and L449F mutations was 13 days posttransfection (Fig. 2a). A similar growth kinetics pattern was observed in the PR with the L90M mutation. The PR with the L90M mutation was selected as a representative PI resistance-associated mutation that has little effect on viral fitness. The viruses with the L90M mutation and both the wild-type Gag sequence and the L449F CSM demonstrated peak RT activity on day 10, whereas the viruses with the A431V mutation and that with the A431V and L449F mutations demonstrated peak RT activities on day 13 (Fig. 2b).

The growth pattern of the clone with the D30N, N88D, and L90M mutations was different from that of the viruses with the wild-type sequence or the PR with the L90M mutation. Viruses with PI resistance associated with the D30N, N88D, and L90M mutations were selected as representatives of viruses with severely impaired PR activities. Viruses with the D30N, N88D, and L90M mutations and the wild-type Gag sequence demonstrated a substantial delay in growth compared to the time of growth for the HXB2 wild type. The peak RT activity of the clone with the D30N, N88D, and L90M mutations was observed at day 17 posttransfection, 7 days after the times of peak activity for the wild-type virus and the virus with the L90M mutation. For the virus with impaired PR activity, the L449F CSM slightly improved the growth kinetics in the presence of the D30N, N88D, and L90M mutations; and peak RT activity was shifted to a point 2 days earlier than that for the virus with the wild-type Gag sequence. It appears that the contribution of L449F to viral fitness is essential in viruses with less fit PRs. In contrast, the A431V CSM was not beneficial to viral replication in viruses with the D30N, N88D, and L90M mutations. Viruses with the A431V mutation or the A431V and L449F mutations demonstrated reduced levels of growth (Fig. 2c).

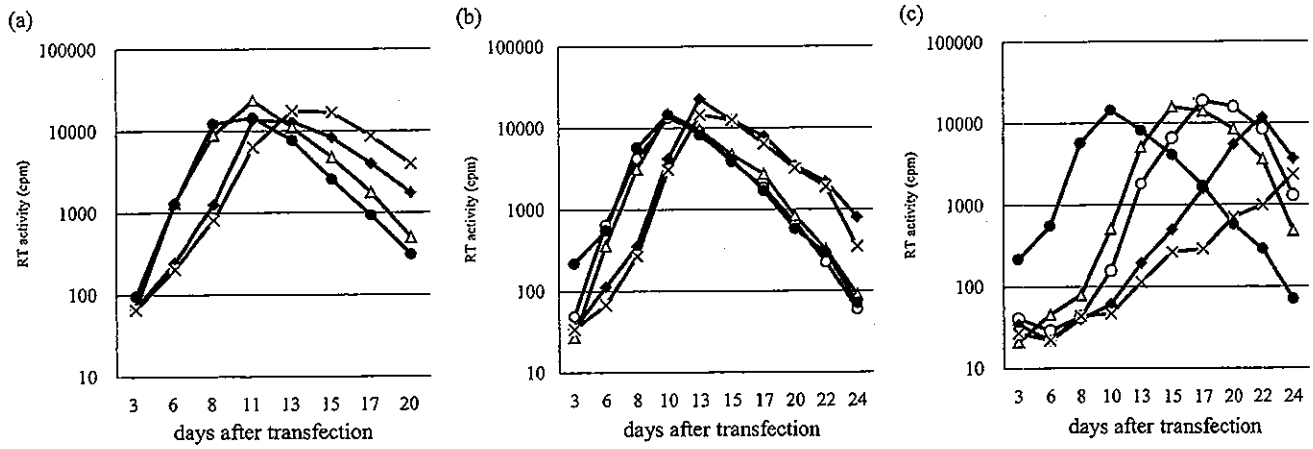

FIG. 2. Replication kinetics of viruses with artificially induced PI resistance. Replication kinetics result in three different PRs: (a) wild-type PR; (b) a PR with an L90M mutation; and (c) a PR with D30N, N88D, and L90M mutations. Ten micrograms of recombinant virus DNA was transfected into 5×10^6 MT-2 cells by electroporation on day 0, and virus cultures were monitored for RT activity every 2 to 3 days. Solid circles, wild-type virus with wild-type Gag and PR; open circles, virus with wild-type Gag; open triangles, virus with the p1/p6 L449F CSM; solid diamonds, virus with p7/p1 A431V CSM; multiplication signs, virus with both the A431V and the L449F CSMs.

The p1/p6 L449F CSM improved the replication of viruses with the D30N, N88D, and L90M mutations, whereas the p7/p1 A431V CSM was deleterious to virus replication in all three viruses tested.

Sequence profiles of selected patient-derived Gag-PR recombinant viruses with CSMs and non-CSMs. Samples from 43 patients who failed nelfinavir-containing regimens were selected from among the samples sent to the AIDS Research Center, National Institute of Infectious Diseases. Clones were carefully selected according to their drug resistance mutation patterns and Gag CSM patterns so that the population carrying the required CSM and PI resistance-associated mutations was represented. Two clones that had mutation patterns similar to those detected in the molecular clone analyses with viruses with artificially induced PI resistance described above were selected, the three Gag-PR fragments were cloned, and the following recombinant viruses were constructed: (i) a clone with the D30N, N88D, and L90M mutations in the PR sequence and the A431V and L449F mutations in the Gag sequence; (ii) a clone with the D30N, N88D, and L90M mutations in the PR sequence and the L449F mutation in the Gag sequence; and (iii) a clone with the L90M mutation in the PR sequence and the A431V mutation in Gag sequence. The major difference between the patient-derived clones and the clones with artificially induced PI resistance was that the Gag-PR fragments of the patient-derived clones had additional mutations in the Gag and PR regions. The sequences of the three Gag-PR fragments are shown in Fig. 3a and b. Clone 1-1 had both the A431V and the L449F CSMs, and there was one base deletion and 15 substitutions in the non-cleavage site and an APP duplication after position 459. The PR region contained three major mutations for PI resistance (D30N, M46I, and L90M) and seven minor mutations for PI resistance (L10I, L23I, I54V, L63P, A71T, V77I, and N88D). In addition, there were three mutations (K43T, I72E, and I97L) that are not recognized as drug resistance-associated mutations. As we could not determine the baseline sequence before PI treatment, we could not determine the source of the last three mutations, that is, whether they occurred by natural polymorphism or were introduced during PI treatment. However, we were able to obtain the sequence data for virus obtained in the early PI treatment phase, prior to the emergence of mutations for PI resistance. By comparison of the sequence of clone 1-1 with the sequence of the virus obtained at this early time point, we estimated that I72E seemed to be a natural polymorphism and that K43T and I97L probably appeared during PI treatment. One amino acid deletion at position T371 and five substitutions (I401V, E468A, Q474L, R479I, and A487S) appeared in the Gag region after PI resistance acquisition.

Since clone 1-2 originated from the same patient sample as clone 1-1, the Gag sequence of clone 1-2 was nearly identical to that of clone 1-1. The differences between these clones were that clone 1-2 did not have the A431V CSM or the I401V non-CSM. We found more differences in the PR regions between clones 1-1 and 1-2. Fewer PI resistance-associated mutations accumulated in clone 1-2. Compared to the sequence of clone 1-1, clone 1-2 did not have the M46I, L23I, or I54V mutations. In addition, two mutations not related to resistance, K43T and I62V, were missing from clone 1-2.

The Gag sequence pattern of clone 2-2 selected from the sample from the second patient was quite different from those of clones 1-1 and 1-2. In addition to the A431V CSM in the Gag cleavage site, the clone had two additional mutations within the R380K (p2/p7) and P453L (p1/p6) cleavage sites. The clone also had 10 substitutions in the non-cleavage site. We were able to obtain a plasma sample before the initiation of therapy with the PI and determined that the mutations appeared after PI treatment. Four mutations (I389T, P453L, T456S, and P497L) were found to have appeared after PI treatment and resistance acquisition. Two major PI resistance-associated mutations (M46I and L90M) and four minor PI resistance-associated mutations (L10V, L63P, A71V, and G73S) were observed in the PR region. Eight additional mutations not related to resistance were also found in the PR region.

Using the three patient Gag-PR clones as templates, we successfully constructed recombinant viruses with four differ-

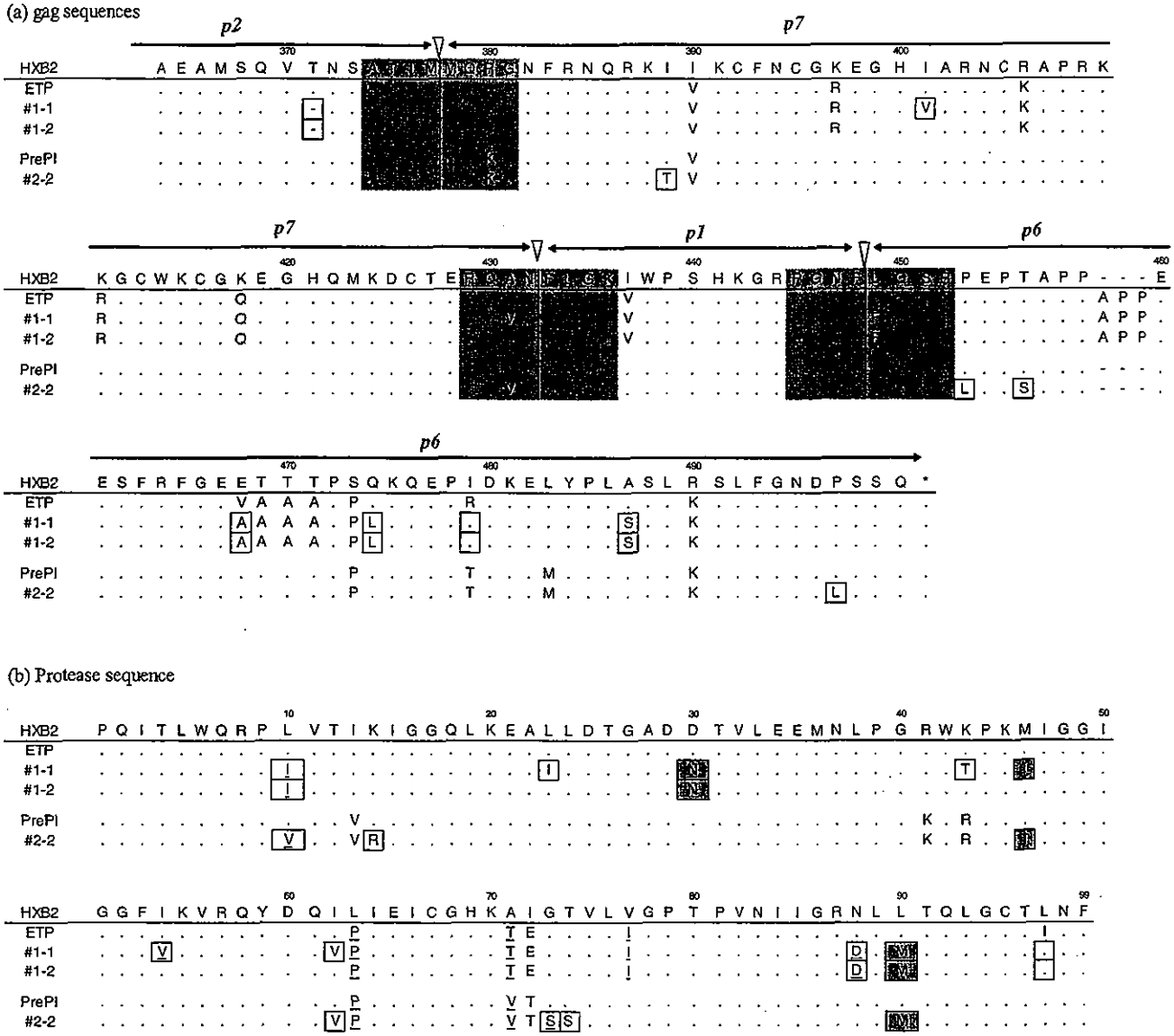

FIG. 3. Gag and PR sequences of patient-derived clones. (a) Gag sequences of three patient-derived clones (clones 1-1, 1-2, and 2-2) aligned according to the sequence of reference strain HXB2. To clarify that the mutation appeared at the time of treatment, the baseline sequences are also demonstrated. Sequence data for the virus present prior to treatment were not available for the clones from the first patient (clones 1-1 and 1-2). Therefore, reference is made to the sequence data for virus obtained at an earlier time point of treatment (ETP), a time point at which no PI resistance-associated mutations had emerged. These data are used for comparison to the baseline sequence. Sequence data for the virus present prior to the initiation of PI treatment (PrePI) were obtained for the virus from the second patient (clone 2-2). The shaded areas indicate the cleavage sites, and open triangles indicate the cleavage points. Mutations that appeared after treatment are highlighted in open squares. (b) PR sequences of the three patient-derived clones (clones 1-1, 1-2, and 2-2) aligned according to the sequence of reference strain HXB2. For the clone from the first patient; sequence data for virus obtained at an earlier time point of treatment were used as the baseline sequence, and for the clone from the second patient, sequence data for the virus present prior to the initiation of PI treatment were used as the baseline sequence. The shaded loci indicate major PI resistance-associated mutations, and underlining indicates minor mutations. Mutations that appeared after PI treatment are highlighted in open squares.

ent combinations of Gag and PR fragments, types GP, P, P^{+c}, and GP^{-c}.

Contributions of CSMs and non-CSMs and highly significant relationship between CSM and non-CSM in the virus fitness recovery process. To evaluate the contributions of the A431V and L449F CSMs and to clarify the relevance of non-CSMs, four different types of recombinant virus were constructed by using the three patient-derived Gag-PR clones as templates. The viruses were cultured independently, and the growth kinetics were monitored. The results of the independent culture studies are shown in Fig. 4. For clone 1-1, the GP type was the most actively growing virus, and the P^{+c} type was the second most actively growing virus. The P type and GP^{-c} types did not grow very well and showed only slight increases in RT activity from the baseline (Fig. 4a). Thus, the level of growth of the four 1-1-derived recombinant viruses was, in

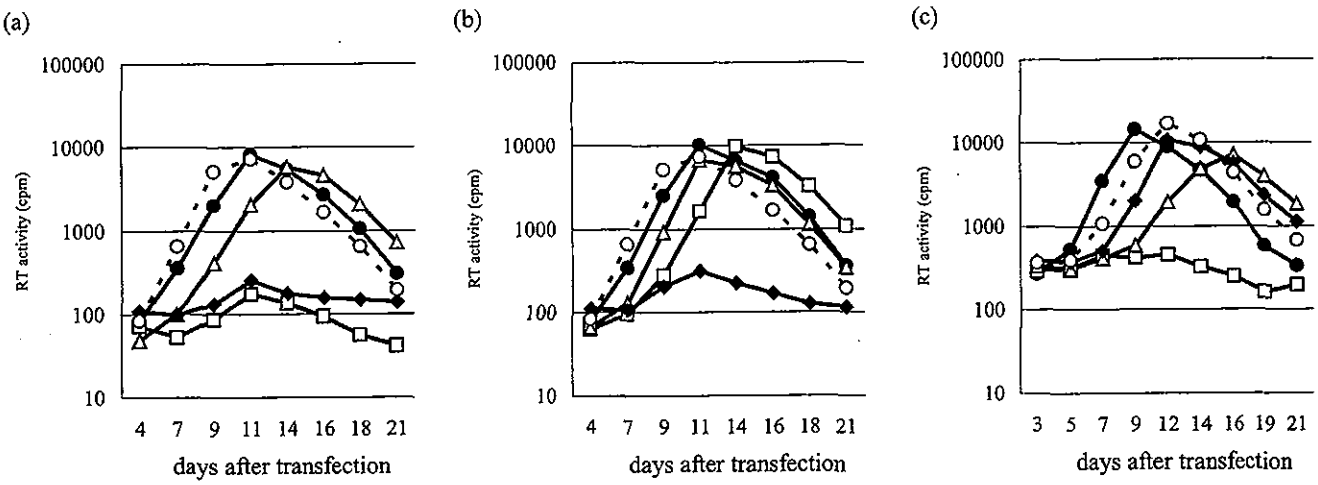

FIG. 4. Replication kinetics of recombinant viruses with patient-derived Gag-PR sequences in independent cultures. The replication kinetics of the recombinant viruses with patient-derived Gag-PR sequences were evaluated by using independent cultures. The results for three different patient-derived Gag-PR sequences are shown: (a) clone 1-1, (b) clone 1-2, and (c) clone 2-2. Ten micrograms of recombinant virus DNA was transfected into 5×10^6 MT-2 cells by electroporation on day 0, and virus cultures were monitored for RT activity every 2 to 3 days. Open circles, HXB2 wild-type control virus; solid circles, GP-type recombinants; open squares, P-type recombinants; open triangles, P^{+c}-type recombinants; solid diamonds, GP^{-c}-type recombinants.

order from the most to the least active, $GP > P^{+c} > P = GP^{-c}$. A competition culture was performed to confirm the replication capacities of the GP and P^{+c} types. As shown in Fig. 5a, the GP and P^{+c} types grew in similar proportions up to day 14, but the GP type dominated after day 21 and comprised 70 to 80% of the virus population in the supernatant. Thus, the GP type had better growth than the P^{+c} type, and the result was consistent with that of the independent culture study. In clone 1-1, two CSMs were important for fitness recovery, as the P^{+c} type grew better than the P type. However, by comparison of the GP and P^{+c} types, the CSMs were not sufficient and 17 additional non-CSMs were required for full recovery of viral fitness. Surprisingly, the combination of 17 non-CSMs observed in clone 1-1 seemed to be deleterious to viral fitness without CSMs, since the GP^{-c} type failed to grow. It appears that this combination of non-CSMs was functional only in the context of the A431V and L449F mutations.

Both the GP and the P^{+c} types of clone 1-2 (another clone from the same patient from which clone 1-1 was derived) grew efficiently. The P type of this clone also grew, but the GP^{-c} type failed to grow (Fig. 4b). Thus, the order of fitness for the clone 1-2-derived recombinants was, in order from the most to the least fit, was $GP = P^{+c} > P > GP^{-c}$. To confirm this order, three pairs of competition assays with the GP type versus the P type, the GP type versus the P^{+c} type, and the P^{+c} type versus the P type were performed (Fig. 5b to d). In the competition with the GP and P^{+c} types, the growth of the two viruses was similar during the culture period. In the competition assays with the GP and P types, the GP type dominated after 14 days. In the competition with the P^{+c} type and the P type, the P^{+c} type was always dominant and comprised 90 to 100% of the virus population. Thus, the results were consistent with those of the independent cultures. A reduced level of replication of clone 1-2 was observed, and the L449F CSM improved the growth kinetics up to a level similar to that for the GP type. The combination of 16 non-CSMs without the L449F mutation was deleterious to replication in this clone as well.

Clone 2-2, which was derived from a different patient, demonstrated a kinetics pattern different from those of the other two clones. The GP type was the most actively growing virus, and this finding was consistent with those for clones 1-1 and 1-2 (Fig. 4c). Different from the other two clones, the GP^{-c} type was the next most actively growing, followed by the P^{+c} type. The P type failed to grow. Thus, the order of fitness, from the most to the least fit, was $GP > GP^{-c} > P^{+c} > P$. The A431V CSM was critical to the recovery of fitness for this clone but was not sufficient for full recovery, and additional non-CSMs were required. To confirm the order of fitness, three pairs of competition assays were performed: the GP type versus the P^{+c} type, the GP versus the GP^{-c} type, and the GP^{-c} type versus the P^{+c} type (Fig. 5e to g). In the competition assay with the GP type versus the P^{+c} type, the GP type dominated after 7 days, and 80 to 90% of the virus population was of this type. In the competition assay with the GP type versus the GP^{-c} type, the GP type was always found at a higher percentage throughout the culture period, but the GP^{-c}-type virus was also observed in 20 to 40% of the virus population. It appears that the GP type had a slight competitive advantage compared to that for the GP^{-c} type. In the competition assay with the GP^{-c} type versus the P^{+c} type, the GP^{-c} type grew better than the P^{+c} type and more than 90% of the clones were of the GP^{-c} type at day 28. Thus, the data were consistent with those of the independent culture study. In clone 2-2, Gag mutations were critical for viral replication. CSM A431V and a combination of 10 non-CSMs both had a positive effect on the recovery of viral fitness and appeared to be most effective when CSMs and non-CSMs coexisted.

DISCUSSION

The complementary relationship of Gag mutations within substrate sites, i.e., CSMs, with PI resistance has been reported in many studies (10, 14, 17, 24, 44). However, our analysis of patient-derived Gag sequences showed that CSMs are not the

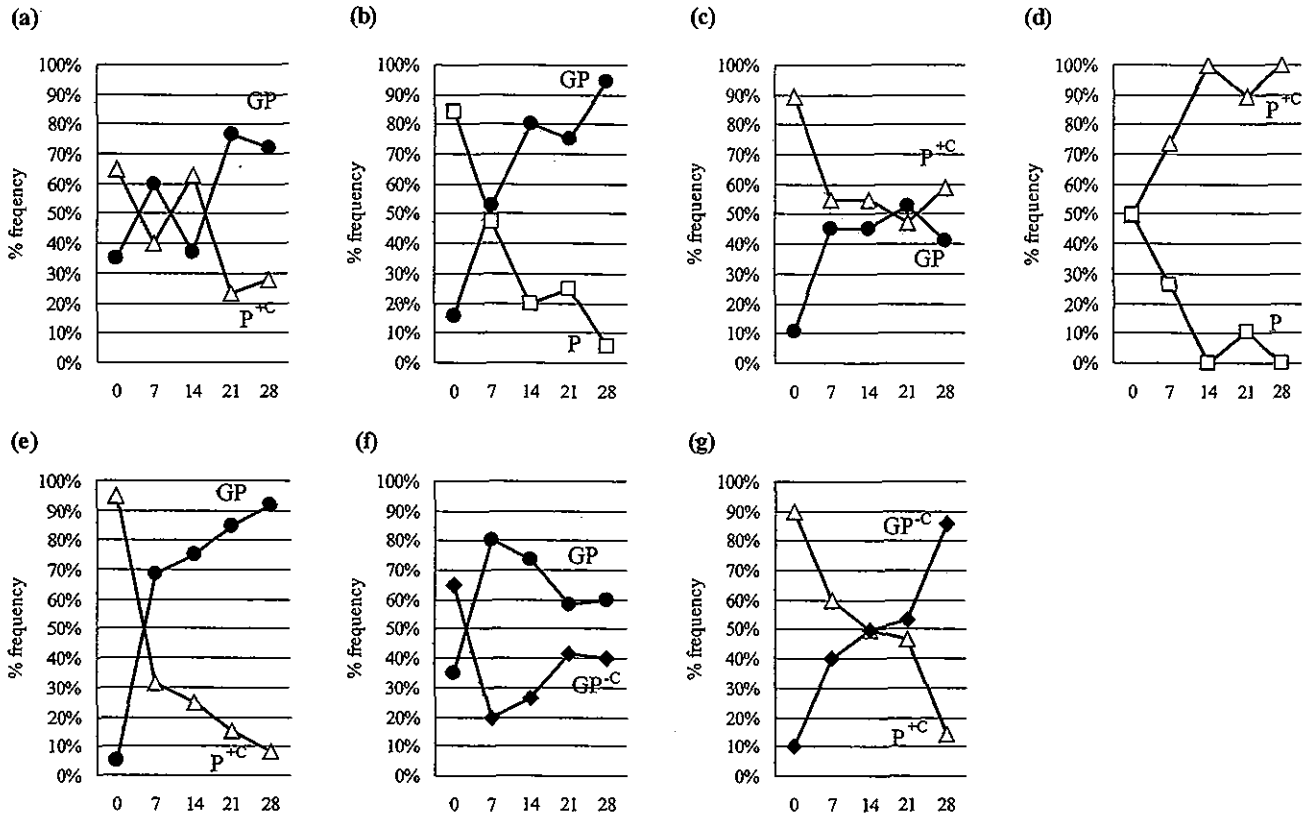

FIG. 5. Replication kinetics of recombinant viruses with patient-derived Gag-PR sequences in competition culture. To confirm the results of the independent cultures, competition assays were performed with the cultures. The results of seven competitions assays are shown. (a) Clone 1-1 of the GP versus clone 1-1 of the P^{+c} type; (b) clone 1-2 of the GP type versus clone 1-2 of the P type; (c) clone 1-2 of the GP type versus clone 1-2 of the P^{+c} type; (d) clone 1-2 of the P^{+c} type versus clone 1-2 of the P type; (e) clone 2-2 of the GP type versus clone 2-2 of the P^{+c} type; (f) clone 2-2 of the GP type versus clone 2-2 of the GP^{-c} type; (g) clone 2-2 of the GP^{-c} type versus clone 2-2 of the P^{+c} type. Solid circles, GP type; open squares, P type; open triangles, P^{+c} type; solid diamonds, GP^{-c} type.

only mutations that appear together with PI resistance-associated mutations, but additional Gag mutations accumulated in non-CSMs, outside the cleavage sites. In this study, we focused on the mutations found outside of the cleavage sites and attempted to clarify their importance in terms of the recovery of viral fitness. Some of the non-CSMs may have existed as baseline Gag mutations prior to ART, and some of them may have emerged after the initiation of ART.

We started the study by confirming the impact of CSMs on viral fitness in PI-resistant clones, and we selected two PI resistance-associated mutation patterns, the D30N, L90M, and N88D mutation pattern, which is representative of a highly impaired PR, and the L90M mutation pattern, which is representative of a fairly impaired PR. We constructed several recombinants with different CSM patterns and compared their replication kinetics. From the results of these experiments, we verified that the p1/p6 L449F CSM has the potential to improve the fitness of the virus with the D30N, L90M, and N88D PI resistance-associated mutations. We did not see a significant positive effect of L449F in the virus with the L90M mutation or the virus with wild-type PR, nor did the mutation hinder replication of the viruses.

We observed different patterns of kinetics among viruses with the p7/p1 mutation. This mutation has also been reported to improve viral fitness, especially with regard to M46IL PI resistance-associated mutations (8). Indeed, patient-derived clones 1-1 and 2-1, which had the A431V mutation, also had the M46I mutation in the PR gene. However, for wild-type virus and viruses with the L90M mutation and the D30N, N88D, and L90M mutations tested in these studies, the p7/p1 mutation demonstrated deleterious effects on virus growth. A similar negative impact of the A431V mutation on viral growth was also reported in two previous studies (14, 29). All six clones with the A431V mutation demonstrated slower viral replication compared to the rate of replication of clones without the A431V mutation. The data also support a significant linkage and the synergistic evolution of the A431V and M46IL mutations.

Interestingly, the growth kinetics of viruses with p7/p1 and p1/p6 double cleavage sites were the worst for wild-type viruses and viruses with the D30N, N88D, and L90M mutations. It seems that there might be interference between these two CSMs, and introduction of additional Gag and PR mutations might be required to overcome this negative effect.

To evaluate the contributions of CSMs and non-CSMs in viral replication, we compared the replication kinetics of four types of recombinant viruses. We performed two different types of experiments. One was an independent culture assay in