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Identification of drug-related genotypic changes in HIV-1 from serum using the selective polymerase chain reaction

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Abstract

We attempted to detect drug-related HIV-1 *pol* gene mutations by selective polymerase chain reaction (PCR) using both proviral DNA and viral RNA isolated from patients (pts) with AIDS or ARC receiving antiretroviral therapy. Peripheral blood mononuclear cell (PBM)-associated proviral DNA and serum-derived viral RNA were obtained from eight patients before and after receiving an alternating regimen of AZT and ddC for 15–41 months or ddI monotherapy for 12–26 months. These specimens were examined for the presence of mutations at positions 70, 74, 215 and 219. We noted that selective PCR results can be ambiguous depending on the quantity of DNA template employed. We, therefore, used the minimal quantity of DNA templates that yielded evaluable PCR products in this study. For all the eight pairs of pre- and post-therapy proviral DNA samples, selective PCR results agreed with independently determined nucleotide sequences. Results of reverse transcription of serum-derived viral RNA followed by selective PCR differed in some cases from those using the proviral DNA. In particular, the use of serum viral RNA appeared to allow earlier detection of changes in drug-related mutations than the use of PBM-associated proviral DNA. We conclude that (i) selective PCR using the minimum and sufficient number of PBM-associated proviral DNA and serum viral RNA copies successfully detects the presence of known *pol* gene mutations; (ii) drug-related mutations may be distinguished earlier in virions in serum (or plasma) than in proviral DNA in PBM; and (iii)

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quantification of HIV-1 prior to selective PCR may be an important component in monitoring the therapy of HIV-1 infection.

Keywords: HIV-1; Selective PCR; Proviral DNA; Viral RNA

1. Introduction

Anti-HIV therapy employing the currently available antiretroviral agents appears to be limited by its toxicity and apparent inability to ultimately prevent progression of HIV-1-related diseases (Mitsuya et al., 1991; Yarchoan et al., 1991; Mitsuya and Yarchoan, 1994; Balzarini and De Clercq, 1994). The emergence of HIV-1 variants less susceptible to reverse transcriptase inhibitors, initially described for AZT (Larder and Kemp, 1989) and subsequently for ddI (St. Clair et al., 1991), may be a significant factor in the incomplete clinical effectiveness of these agents (Richman, 1994). In fact, there is a growing body of literature to correlate the emergence of HIV-1 variants with reduced susceptibility to antiretroviral drugs with clinical deterioration in patients receiving these agents (Kozal et al., 1993; Montaner et al., 1993; Ogino et al., 1993; Tudor-Williams, 1992; Tudor-Williams et al., 1992). Thus, the identification of mutations known to be associated with drug-resistance has become important both in the development of anti-HIV agents and in the treatment of patients with HIV-1 infection.

Larder and his co-workers introduced a method to detect mutations in the *pol* gene which employs a nested polymerase chain reaction (PCR) (Larder et al., 1989; Larder et al., 1991). This technique, termed selective PCR, is now being considered as a method to screen samples from HIV-1-infected individuals receiving certain antiretroviral agents for the presence of known nucleotide changes in the genetic code of HIV-1. However, the reproducibility and clinical usefulness of the selective PCR method remain to be established. In the present study, we attempted to improve the selectivity and reliability of the selective PCR method by using serially diluted samples and known numbers of HIV-1 virions. We also asked whether cell lysate proviral DNA or serum (or plasma) viral RNA allowed earlier detection of drug-related *pol* gene mutations.

2. Materials and methods

2.1. Virus

Cryopreserved peripheral blood mononuclear cells (PBM) ($1-2 \times 10^6$) isolated from heparinized blood of patients with advanced HIV-1 infection before and after receiving alternating AZT/ddC therapy for 15–41 months or ddI monotherapy for 12–26 months as described before (Shirasaka et al., 1993) were cocultured for 7 days with phytohemagglutinin-activated PBM ($1-2 \times 10^6$) obtained from healthy blood donors. After 7 days of culture, cell lysates were prepared from the PBM population by incubating 7.5×10^6 PBM/ml in lysis buffer containing 0.4% NP-40 (Sigma, St. Louis, MO), 0.4% Tween-20 (Sigma, St. Louis, MO), 1 mg/ml of Proteinase-K (Boehringer-Mannheim, Germany), 50 mM KCl, 10 mM Tris-HCl, and 2 mM $MgCl_2$ at 56°C for 2 h and then inactivating the proteinase by incubating the lysate at 95°C for 15 min. The cell

Table 1
Amino acid substitutions identified in HIV-1 isolates studied

Patient	HIV-1 isolate	Amino acid substitutions
100	pre-therapy	none *
	post-therapy	Asp-67 → Asn(8/8); Lys-70 → Arg(8/8); Thr-215 → Tyr(6/8); Lys-219 → Gln(8/8)
101	pre-therapy	none *
	post-therapy	Asp-67 → Asn(8/8); Thr-69 → Asp(8/8); Lys-70 → Arg(8/8); Thr-215 → Phe(8/8); Lys-219 → Gln(8/8)
103	pre-therapy	none *
	post-therapy	Ala-62 → Val(8/8), Val-75 → Ile(8/8); Phe-77 → Leu(8/8); Phe-116 → Tyr(8/8), Gln-151 → Met(8/8)
200	pre-therapy	none *
	post-therapy	Leu-74 → Val(8/8)
201	pre-therapy	Ile-142 → Thr(8/8)
	post-therapy	Leu-74 → Val(8/8); Ile-142 → Thr(8/8)
203	pre-therapy	Met-41 → Leu(4/4); Thr-215 → Tyr(4/4)
	post-therapy	Met-41 → Leu(8/8); Leu-74 → Val(8/8); Thr-215 → Tyr(7/8)
204	pre-therapy	Asp-67 → Asn(1/4); Lys-70 → Arg(3/4)
	post-therapy	Thr-215 → Tyr(8/8)
205	pre-therapy	Lys-70 → Arg(1/4)
	post-therapy	none *

An amino acid after an arrow for each isolate indicates a variation from the consensus sequence 1 of HIV-1 POL. For example, the post-therapy HIV-1 isolate from patient 100 had an Asp → Asn substitution at codon 67 in 8 out of 8 clones examined (designated Asp-67 → Asn(8/8)). Only selected mutations in the *pol* gene are listed in this table. * none: Mutations which are not reported to be associated with therapy with nucleosides are not listed. See Shirasaka et al., 1993 for more details.

lysates were then stored at −20°C until use. HIV-1 virions from serum samples (1 ml), which had been obtained at the same pre- and post-therapy time points and stored at −70°C until use, were isolated by ultracentrifugation and subjected to RNA extraction (see below). Amino acid substitutions identified in each viral population, as compared with the consensus HIV-1 sequence, are illustrated in Table 1.

2.2. *Selective PCR method*

Proviral DNA of cell lysates and viral RNA derived cDNA were subjected to an initial PCR amplification of a polymerase-coding region of the HIV *pol* gene (742 bp fragment) by 35 amplification cycles using a DNA thermal cycler (Perkin-Eimer Cetus) and primers L1M (5′-TTGCACTTTAAATTTTCCCATTAG-3′) and AS62 (5′-GGCTGTACTGTCCATTTATCAGGA-3′) with a PCR annealing temperature of 45°C in reaction buffer containing 25 mM KCl and 2.0 mM $MgCl_2$. The initial amplification product was diluted 1:10 or greater and then selective PCR (30 amplification cycles) was performed using previously described primers for codons 70, 74, 215, and 219 (Larder et al., 1991, St. Clair et al., 1991). Reaction conditions were as follows: (i) the codon 70 reaction buffer included 2.0 mM of $MgCl_2$ and 25 mM of KCl, with a PCR annealing temperature of 45°C; (ii) the codon 74 reaction buffer included 1.5 mM of

$MgCl_2$ and 50 mM of KCl, with a PCR annealing temperature of 45°C; (iii) the codon 215 reaction buffer included 1.5 mM of $MgCl_2$ and 25 mM of KCl, with a PCR annealing temperature of 56°C; (iv) the codon 219 reaction buffer included 1.5 mM of $MgCl_2$ and 25 mM of KCl, with a PCR annealing temperature of 54°C. Each 100 μl reaction mixture for PCR amplification included 50 pmol of each primer, 200 μM of dATP, dGTP, dTTP, and dCTP (Promega, Madison, WI), 1 μg of herring sperm DNA (Pharmacia, Milwaukee, WI), and 5 units of AmpliTaq DNA Polymerase (Perkin-Elmer, Norwalk, CT). Eight microliters of the reaction mixture containing PCR product was then loaded on a 1.8% agarose gel in Tris-borate-EDTA buffer (pH 8.4) and electrophoresed at 80 volts for 45 min. DNA was stained with ethidium bromide for ultraviolet light detection.

2.3. PCR with quantitated pol gene template

Plasmid DNA used for the PCR standardization was obtained by previously described methods using PBM from each patient as the viral source (Shirasaka et al., 1993). The DNA content of stocks of plasmid with mutations of interest was determined by spectrophotometric measurement at 260 nm wavelength. Based on the measured DNA content and the length of the dsDNA plasmid, the number of copies of dsDNA was calculated. One copy of dsDNA was considered to be two templates for the PCR reaction. Serial dilutions of the stock plasmid DNA were made to provide the appropriate number of DNA template copies in a 10 μl vol. for PCR. Water containing 1 μg/ml of herring sperm DNA was used as a diluent to prevent loss of template DNA at the lower concentrations.

2.4. Determination of RNA content in HIV-1 virions (RNA-PCR)

The amount of viral RNA in each viral preparation was determined by using PCR following reverse transcription of viral RNA (RNA-PCR) as previously described (Aoki-Sei et al., 1992) with some modifications. A pair of *pol* gene primers was employed to amplify HIV-1 sequences: AS-61 (5′-ATTGGGCCTGAAAATC-CATACAAT-3′) and AS-62, which yielded a 578 base-pair PCR product. Each serum sample (1 ml), after 10-fold dilution with phosphate-buffered saline (PBS) containing 1% (WIV) DNase/RNase-free bovine serum albumin fraction V (Miles Laboratories, Kankakee, IL), was ultracentrifuged at 30 000 × g for 1 h at 4°C in a Heraeus Sepatech high speed centrifuge. Supernatant in each sample was carefully removed and the pelleted virus was resuspended in 50 μl of DNase/RNase-free Tris-HCl-buffered saline (10 mM Tris, 150 mM NaCl, pH 7.5). RNA was then extracted using the acid guanidinium thiocyanate-phenol-chloroform method described by Chomczynski and Nicoletta (Chomczynski and Nicoletta, 1987). The purified viral RNA solution (50 μl) was treated with RNase-free DNase I and was subjected to ethanol precipitation following inactivation of the DNase I activity. Precipitated RNA was dissolved in reverse transcription reaction mixture containing the downstream primer, AS-62, transcribed by Moloney murine leukemia virus reverse transcriptase (UIBCO BRL, Gaithersburg, MD) for 1 h, followed by an incubation at 95°C for 5 min to inactivate

reverse transcriptase. A portion (6 μl) of the reaction mixture containing cDNA, 50 pmol of primers AS61 and AS62, and 10^6 cpm of ^{32}P-end-labeled AS61 was subjected to PCR. PCR was performed with 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and polymerization at 72°C for 1 min. DNase-treated RNA samples without the RT reaction were also subjected to PCR to confirm that there was no detectable contaminating HIV-1 DNA.

The amplified DNA was subjected to electrophoresis on a 10% polyacrylamide gel, and the dried gel was exposed to Kodak X-OMAT film at room temperature for 1 h. The density of the expected size band on the film was measured by densitometer and analyzed by using the software Quantity One (PDI, Huntington, Station, NY). A HIV-1_{LAI} viral preparation whose particle number per ml had been determined by electron microscopy was used as a standard.

3. Results

3.1. Low and sufficient numbers of DNA template yields more reliable selective PCR results

We noted that the use of unquantified templates in selective PCR could lead to difficulty in discriminating between wild and mutant nucleotide sequences. This became evident when quantified numbers of plasmids were subjected to the selective PCR assay. We found that 10^5–10^{10} copies of plasmid derived from a wild-type virus isolate produced the wild-type configuration for Codon 215 (Fig. 1, upper left panel). However, 10^{11} and 10^{12} copies of the same plasmid produced a 'mixed' configuration, although the profile was still wild-type dominant. Codon 215-mutant plasmid produced the mutant configuration at a range of 10^7 and 10^9; however, the same plasmid preparation generated a mixed configuration at 10^{10} and beyond. It was noted that 10^{12} mutant plasmids produced a 'mixed' configuration of equal amplification product intensity (Fig. 1, upper right panel).

As shown in Fig. 1, lower panel, wild-type and mutant plasmids produced clearcut wild and mutant configurations, respectively, for codon 74 at a range of 10^6 to 10^{10} copies; however, both of the same plasmids produced the 'mixed' configuration when 10^{11} and 10^{12} copies were used. Thus, a low but sufficient quantity of template appeared to yield a more selective and reliable PCR result. In particular, with the codon 215 and 74 selective PCR conditions described, 10^7 to 10^8 template copies would appear to be an appropriate number of template copies to use.

Similar studies using plasmids containing other mutations demonstrated that selective amplification of the wild 70 codon site occurs when 5×10^5 to 5×10^7 copies were used, while the mutant 70 codon site was selectively amplified at a range of 5×10^4 to 5×10^7 copies. The wild 219 codon site was selectively amplified at a range of 5×10^8 to 5×10^{11} copies while the mutant 219 codon site was selectively amplified at a range of 5×10^7 to 5×10^{10} copies. These findings suggest that the configuration of selective PCR products is influenced by the discriminatory range and asymmetry of amplification efficiency for each codon's primer sets.

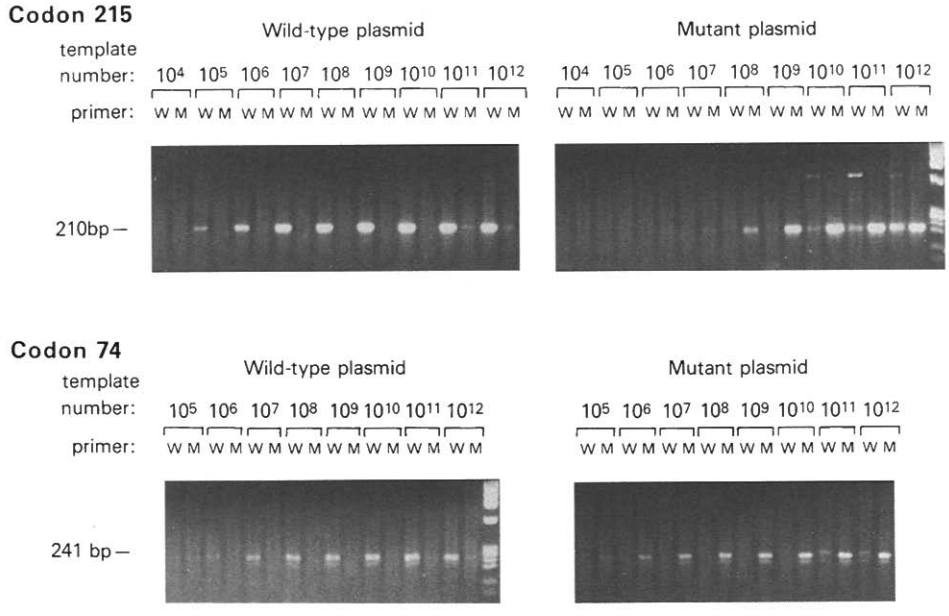

Fig. 1. Selective PCR product configuration with increasing numbers of plasmid DNA templates. The number of plasmids was determined by spectrophotometric analysis of solutions of the 4.5 kb plasmid preparations. Selective PCR was performed using 10^4 to 10^{12} plasmids to discriminate between wild-type (W) and mutant (M) residues at codons 215 and 74. Note that increasing the number of plasmids results in nonspecific product configurations at higher template number for codons 215 and 74. The ϕX174/*Hae*III DNA size marker (Promega, Madison, WI) is also shown.

3.2. Selective PCR product configurations differ significantly depending on numbers of virions used as templates

In order to examine the effect of varying viral genetic template numbers on the PCR product configuration, we performed selective PCR using cell-free virions after quantification of the virus using PCR combined with reverse transcription of viral RNA (RNA-PCR) (Aoki-Sei et al., 1992). When viral cDNA obtained through reverse transcription of viral RNA was subjected to the initial PCR followed by dilutions of the PCR product by 100-fold (H) and 10 000-fold (L), quite different selective PCR product configurations were obtained (Fig. 2). For example, serum samples from patients 1, 3, 6, and 7 yielded either the same configuration (in both dilutions in patients 3 and 7) or a failure of product signal when 10 000-fold diluted initial PCR product was used for the selective PCR (patients 1 and 6). This suggests that the quantity of cDNA employed at the 1:100 dilution allowed a reliable interpretation of the selective PCR product configuration. In contrast, patient samples 2, 4, and 5 demonstrated that the use of the lower template number (in the sample diluted 10 000-fold) produced a different amplification configuration compared with the higher template number (in the sample diluted 100-fold). This data suggests that the use of a low but sufficient quantity of viral DNA or cDNA provides more readily interpretable results.

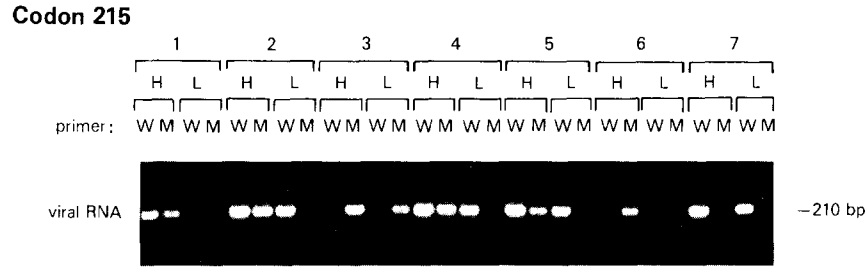

Fig. 2. Different selective PCR product configurations depending on numbers of virus particles. Each cDNA obtained through reverse transcription of viral RNA isolated from each of seven patients was subjected to PCR (initial PCR). Products from the initial PCR were diluted 100-fold (H) and 10000-fold (L) and subjected to selective PCR to discriminate between wild-type (W) and mutant (M) nucleotides at codons 215. Number of virus particles in 5 μl samples subjected to initial PCR were: 1, 1.1×10^3; 2, 3.2×10^5; 3, 1.2×10^5; 4, 2.5×10^5; 5, 1.4×10^5; 6, 4.8×10^4; 7, 4.8×10^4. Note that different dilutions of the initial PCR products can produce different selective PCR product configurations.

3.3. Efficiency of selective PCR varies depending on primer pairs used

We also noted that the efficiency of the selective PCR varied substantially depending on the primer pairs used. When viral RNA-derived cDNA was used, the selective PCR required as few as 10 virus particles in the initial amplification step to discriminate between wild-type and mutant nucleotides at codon 215 (Fig. 3, upper panel). However, to discriminate between wild-type and mutant nucleotides at codon 74 more than 50 virus particles were required to achieve sufficient amplification (Fig. 3, lower panel). The observed difference in the PCR efficiency as a function of the primer pairs employed suggests that the selective PCR does not provide quantitative results and careful interpretation of amplification products is needed, especially when the 'mixed' PCR product configuration is obtained.

3.4. Selective PCR results are consistent with DNA template nucleotide sequence

We then conducted the selective PCR analysis of viral cDNA generated from serum samples of eight patients which were obtained on the same dates as corresponding PBM-associated proviral DNA samples used for nucleic acid sequencing and selective PCR analysis. Tables 1 and 2 illustrate nucleotide sequences of each viral isolate and the results of selective PCR. Results of the selective PCR analysis of the eight pairs of pre- and post-therapy HIV-1 strains essentially all agreed with the nucleotide sequences (Table 1). However, in several cases, the selective PCR product configuration indicated the presence of both wild and mutant genotypes although nucleotide sequences indicated that the proviral population was wild-type or mutant based on the sequence determination of 6 to 8 clones (Tables 1 and 2 and Fig. 4). It is conceivable that the selective PCR technique may allow detection of minor components in the viral population which may not be readily distinguished when low numbers of viral clones are sequenced. It is also possible that this apparent discrepancy is related to the different (asymmetric) efficiency of selective PCR due to the primer pairs used (Fig. 3). The discrepancy may also be

Table 2
Summary of PCR product configuration when proviral DNA or viral RNA was used as a source of template

Patient	Therapy	Source of Template	Codon 70		Codon 74		Codon 215		Codon 219	
			pre	post	pre	post	pre	post	pre	post
100	AZT + ddC (15 Mo)	DNA	WD	MT	WD	WD	WD	MT	WD	MT
		RNA	WD	WD/MT	WD	WD	WD	MT	WD	WD/MT
101	AZT + ddC (22 Mo)	DNA	WD	WD/MT	WD	WD	WD	MT	WD/MT	MT
		RNA	WD	WD/MT	WD	WD	WD	MT	WD	MT
103	AZT + ddC (41 Mo)	DNA	WD	WD	WD	n.d.	WD	WD	WD	WD/MT
		RNA	WD	WD	WD	n.d.	WD	WD	WD	WD/MT
200	ddI (25 Mo)	DNA	WD	WD	WD	MT	WD	WD	WD	WD
		RNA	WD	WD	WD	MT	WD	WD	WD	WD/MT
201	ddI (20 Mo)	DNA	WD	WD	WD	MT	WD	WD	WD	WD
		RNA	WD	WD	WD	MT	WD	WD	WD	WD
203	AZT → ddI (12 → 26 Mo)	DNA	WD/MT	WD	WD	MT	MT	WD/MT	WD	WD
		RNA	MT	WD/MT	WD	MT	MT	WD/MT	WD/MT	WD
204	AZT → ddI (3 → 18 Mo)	DNA	MT	WD	WD	WD/MT	WD/MT	MT	WD/MT	WD/MT
		RNA	WD/MT	WD	WD	WD/MT	WD/MT	MT	WD	WD
205	AZT → ddI (12 → 20 Mo)	DNA	WD/MT	WD/MT	WD	WD/MT	WD/MT	WD	WD	WD
		RNA	WD/MT	WD	WD	WD/MT	WD	WD	WD	WD

Genotypic changes in proviral DNA and viral RNA shown were determined by selective PCR. The duration of a patient's treatment is indicated in parentheses in the Therapy column. Patients 203, 204, and 205 received AZT treatment for 12, 3, and 12 months, respectively, prior to ddI therapy. The 'pretherapy' (pre) samples for 203, 204, and 205 were obtained at the initiation of ddI treatment. pre: pretherapy; post: posttherapy; WD: wild-type codon; MT; mutant codon; n.d.: not determined.

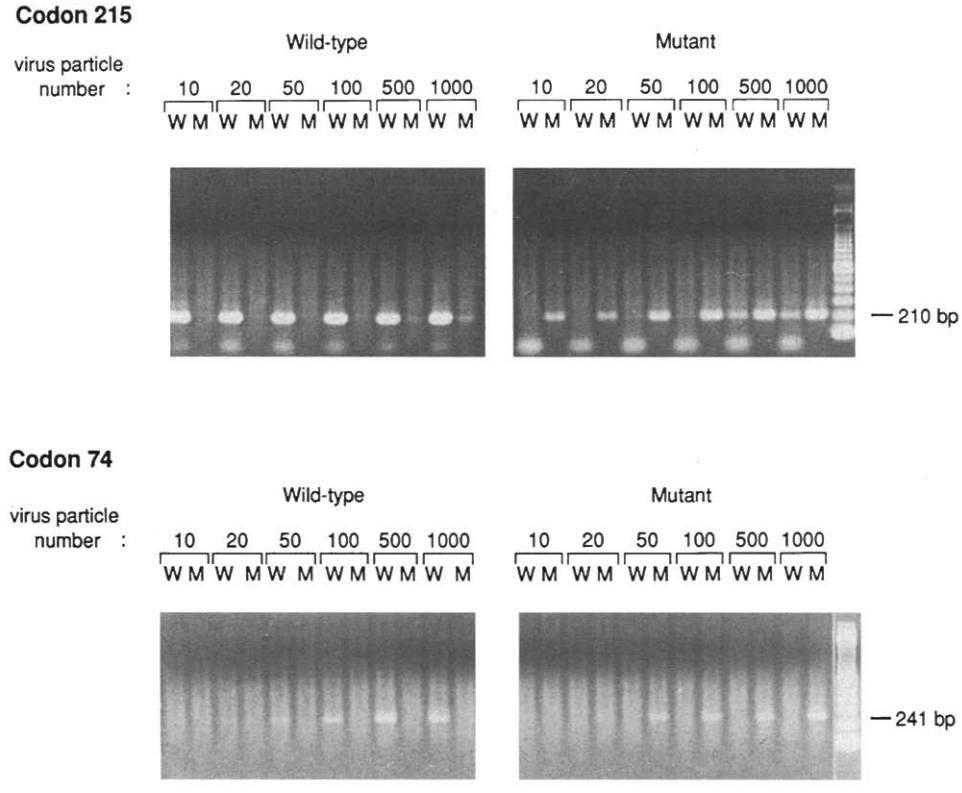

Fig. 3. Selective PCR product configurations with increasing numbers of virus particles. Varying numbers of virus particles were subjected to reverse transcription, initial and selective PCR to discriminate between wild-type (W) and mutant (M) nucleotides at codons 215 and 74. Note that the primer pair for the 215 wild-type codon are more efficient than the primer pair for the 215 mutant codon at the same cDNA template number. The 100 base pair DNA ladder (GIBCO BRL, Gaithersburg, MD) is shown at right.

related to non-specific amplification of the DNA template. However, selective amplification of plasmid DNA with known nucleotide sequences served as a control for each PCR reaction in this study, suggesting that non-specific amplification is unlikely to have occurred.

3.5. Viral RNA may reveal genotypic changes earlier than proviral DNA

In most instances the genotype information derived from viral RNA agreed with the proviral DNA genotype results, but in five cases (patients 100, 101, 203, 204, and 205), the viral RNA codon information differed from that obtained with the proviral DNA (Table 2 and Fig. 4). Patient 203 had received 12 months of AZT monotherapy followed by 26 months of ddI monotherapy. This patient's proviral HIV-1 population showed a codon 215 mutant genotype prior to ddI monotherapy, while it reverted to a predominantly 215 wild genotype after 26 months of ddI monotherapy (Fig. 5, upper left panel).

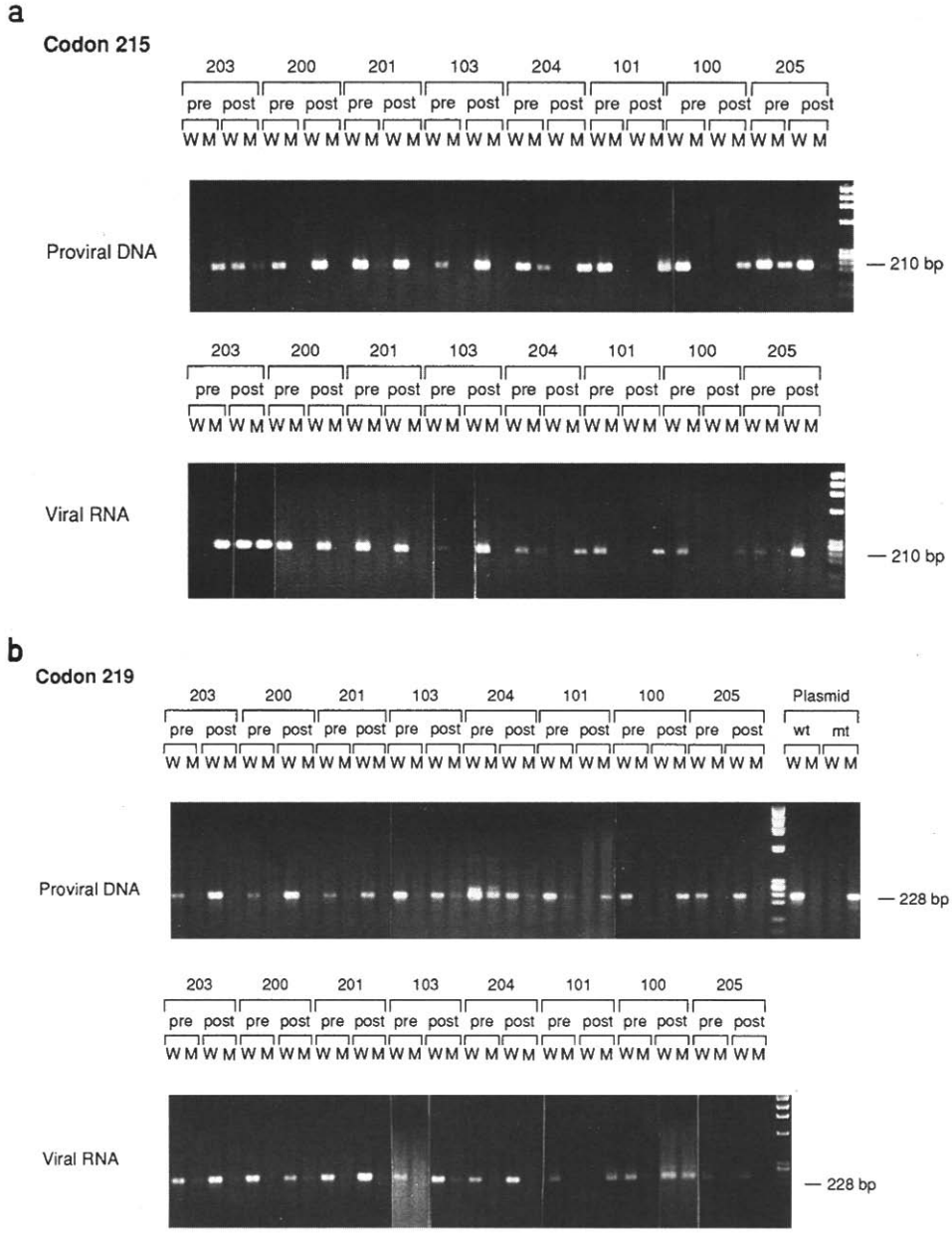

Fig. 4. Proviral DNA can produce different selective PCR product configurations than viral RNA. Patient PBM and serum samples were obtained from eight patients on the same pre- and post-therapy dates. Selective PCR was performed to discriminate between wild-type (W) and mutant (M) nucleotides at codons 215 (a) and 219 (b). While proviral DNA and viral RNA in most cases provided essentially the same genotypic profiles, different configurations were obtained for certain codons in patients 100, 101, 200, 203, 204, and 205. Evidence of codon mutations at the pre-therapy time point for patients 203, 204, and 205 may relate to AZT monotherapy received by these patients before ddI therapy. The ϕX174/*Hae*III DNA size marker is shown at right.

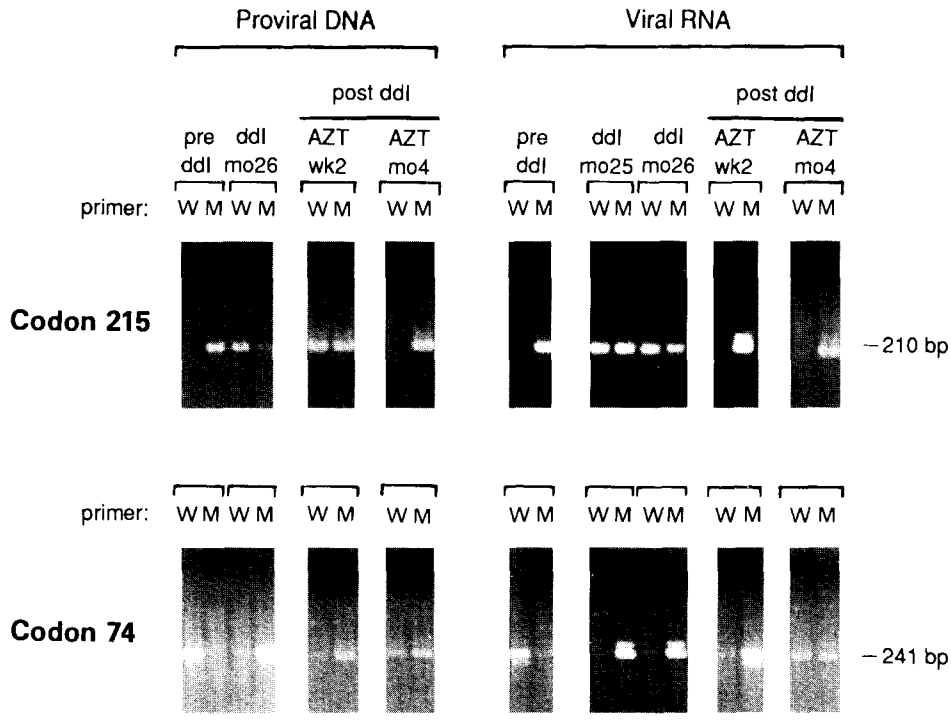

Fig. 5. Viral RNA may demonstrate an early indication of genotypic changes. Viral genotypic changes of patient 203 who received ddI (26 months) and then switched to AZT (patient had received AZT for 12 months prior to initiating ddI monotherapy) are shown. Selective PCR at codon 215 and codon 74 was performed using PBM lysate proviral DNA (at left) and serum-derived viral RNA (at right).

Upon reinstitution of AZT for 2 weeks, this patients's HIV-1 isolate showed a 'mixed' codon 215 genotype configuration when proviral DNA was examined. However, when serum-derived viral RNA samples were examined at the same time point, the patient's HIV-1 population had reverted to a 215 mutant codon predominant configuration (Fig. 5, upper right panel). In patient 204, a shift to wild type was detected earlier in serum-derived RNA than in proviral DNA for codons 70 and 219 (Table 2 and Fig. 4). Similarly, in patient 205, a change in codon 70 was seen earlier in serum-derived RNA. The apparent 'mixed' codon 219 configuration for patient 101 remains unexplained, except for the possibility that the patient received AZT prior to the present study.

Patient 100 demonstrated a 'mixed' PCR product configuration at codon 219 of the viral RNA genome after 15 months of AZT/ddC therapy while proviral DNA indicated a mutant genotype (Fig. 4), as confirmed by proviral DNA nucleotide sequencing. Examination of viral and proviral genomes after 30 months of AZT/ddC therapy demonstrated the wild 219 codon for both viral genetic sources (data not shown), suggesting that the viral RNA reflected a shift of the 219 codon earlier than the proviral DNA. A reversion from a mutant to a wild codon genotype has been noted previously at codon 70 in association with long-term AZT therapy (Boucher et al., 1991).

4. Discussion

In the present study, we examined the reliability and usefulness of the selective PCR technique to detect known *pol* gene mutations in proviral DNA and viral RNA samples. It was found that the specificity of this technique can be substantially affected by the number of DNA templates used. In an attempt to circumvent this problem, we employed a 'dilutional technique' and determined the genetic profile using a minimum number of templates which yielded evaluable PCR products. Such results agreed virtually completely with the *pol* gene nucleotide sequence of each proviral DNA sample. In terms of 'dilutional technique', Shafer and his colleagues recently reported the *pol* gene mutation profiles by performing a 'confirmatory' PCR following 40-fold dilution of the product of the first PCR (Shafer et al., 1994). It should be noted that, in this study, proviral DNA isolated from the coculture of patient PBM and uninfected PBM was used. In this regard, the period of the coculture was only one week and all the PBM (both patient's and uninfected PBM populations) served as the source for proviral DNA. Therefore, the selective PCR data should most likely reflect the proviral DNA of the patient's PBM.

The use of selective PCR for analysis of viral RNA provides a means to analyze the viral genome directly. However, the use of a dilutional technique to produce a discernible selective PCR product is not easily standardized for routine application of the technique. An advantage of using viral RNA directly as the source of viral genetic information is that the sample can first be processed for quantification of virion by means of a reverse transcriptase-based PCR (RT-PCR) technique (Aoki-Sei et al., 1992). The cDNA yielded by this RT-PCR technique can then be utilized in a quantitative fashion to control the input template number for the selective PCR amplification. Thus, it may be advisable that a certain fixed number of virus particles be subjected to the selective PCR assay, so that the combination of viral RNA quantification and selective PCR produces two important pieces of information from the same patient sample. One can also utilize quantified cDNA as standards during the selective PCR technique to control the quality of the amplification results. We are currently employing such an application in the analysis of clinical samples.

An interesting finding in the present study was the detection of the rapid change of the codon 215 from a wild/mutant mixed genotype to a mutant genotype soon after switching to AZT monotherapy in a patient who had previously received AZT followed by ddI monotherapy. In this instance, and similarly for patients 100 and 204, the viral RNA genotypic information presaged the genetic change seen later in the proviral DNA. These data suggest that viral RNA obtained from sera or plasma may represent the actively replicating virus population, while proviral DNA from PBM may reflect the historical repertoire of viral genomes. Genetic variation detected among the actively replicating viral RNA population may be more temporally related to the patient's clinical status than that from the proviral DNA population likely due to the slow turnover of proviral DNA. Indeed, our observation that the genetic change appeared earlier in the viral RNA population than in the proviral DNA population is in concordance with the study by Simmonds et al. (Simmonds et al., 1991) demonstrating that the appearance of genetic mutations in the *env* hypervariable region of plasma viral RNA genomes preceded the occurrence of such sequences in PBM-associated proviral

DNA. Kozal et al. have also found that the codon 215 mutation often appeared in serum HIV RNA before PBM (Kozal et al., 1993), although their patients received only AZT monotherapy. Furthermore, the continued presence of the 215 codon mutant genotype in patient 203 after 26 months of ddI monotherapy is consistent with the findings of St. Clair et al. (St. Clair et al., 1991) in which patients previously treated with AZT were found to maintain the 215 codon mutation after 12 months of ddI monotherapy. Interestingly, the viral RNA demonstrated a rapid shift to the 215 codon mutant genotype while the 74 codon mutation persisted after 4 months of AZT monotherapy, although it is not clear from the PCR results obtained in this study whether the two mutations coexisted in the same virus strain. Although the mutation at codon 41 was not examined in the present study, it may be important in future studies to include the codon 41 mutation among AZT-related mutations monitored by selective PCR.

The short-term coculture of patient PBM with normal donor PBM appears to influence the resulting proviral DNA population (Albert et al., 1992). Meyerhans et al. have also shown that culturing of the virus can disturb the composition of the viral population (Meyerhans et al., 1989). The earlier detection of the reemergence of the codon 215 mutation in viral RNA than in proviral DNA, seen in the case of patient 203, may reflect this possibility. A recent analysis by Smith and her coworkers comparing both plasma and PBM-derived virus also suggests that resistance mutations may be found earlier in plasma-derived HIV RNA than in uncultured PBM proviral DNA (Smith et al., 1993). Our findings support the use of viral RNA as a source for monitoring response to antiretroviral therapy.

Thus, we conclude that (i) selective PCR using a minimum and sufficient number of PBM-associated proviral DNA and serum viral RNA copies successfully detects the presence of known *pol* gene mutations; (ii) drug-related mutations may be distinguished earlier in virions in serum (or plasma) than in proviral DNA in PBM; and (iii) quantification of viral template prior to selective PCR may be an important component of clinical diagnostic studies employing the selective PCR technique.

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