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Nonsense mutations in the *vpr* gene of HIV-1 during in vitro virus passage and in HIV-1 carrier-derived peripheral blood mononuclear cells

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Abstract Long-term, persistent infection by HIV-1 is a prerequisite for the development of AIDS. However, little is known of the determinants required for HIV-1 to cause persistence. We have reported previously that persistent infection of a T cell line by a cytopathogenic strain of HIV-1 became increasingly likely with in vitro serial passage of the virus. DNA sequencing of the persistent strains revealed a nonsense mutation in the *vpr* gene in all isolates tested. Here, we report the development and use of a semi-quantitative PCR method to detect the *vpr* nonsense mutation within populations of virus. Our results show that *vpr* mutants also arise in cells during acute infection and increase progressively with serial passage of the virus. In addition, HIV-1-seropositive individuals were examined and found to carry the same *vpr* nonsense mutation at high frequency in virus-infected PBMC. These data are consistent with a mechanism of HIV-1 persistence in vivo and in vitro in which virus cytopathogenic potential is lost by the build up of nonsense mutations in *vpr*.

Key words: HIV-1; Accessory gene; *vpr*; Persistent infection; Mutation

1. Introduction

Generally, retroviruses are known to mutate at high rates [1–3]. This high mutation rate allows the selection of adapted virus in response to changing environmental conditions. The extensive genetic and antigenic variability of retroviruses also applies to HIV-1, the causative agent of AIDS [4–9]. HIV-1, a member of the lentivirus subfamily of retrovirus, is highly cytopathogenic [10]. Nevertheless, the continued presence of HIV-1 infection has been demonstrated in all HIV-1-seropositive individuals [11]. Thus, HIV-1 can remain clinically silent for a long period in a state of persistent infection, before the onset of AIDS. A characteristic feature of HIV-1 strains recovered during the persistent period is lower cytopathogenicity when compared with strains isolated from AIDS patients [12,13]. Thus, there might be a positive trend towards the selection of non-cytopathogenic or less cytopathogenic HIV-1 during persistent infection of host cells.

The objective in this study was the detection of non-cytopathogenic HIV-1 forms in order to assess their correlation with virus persistence. We showed previously that infection by HIV-1 carrying mutations at *vif*, *vpr*, or *vpu* generates persistent infection, whilst, by contrast, infection by wild-type HIV-1 caused complete killing of the infected cells [14–16]. In addition, we have shown recently that persistent infection can be generated by wild-type HIV-1 following in vitro serial passage and that the rate of persistent infection increased with serial passage [17]. However, persistently infected cells were only obtained after severe cytolysis of most cells [17], indicating that passaged wild-type HIV-1 stocks consist of mixtures, i.e. a major population of cytopathogenic viruses leading to effective cell killing and a minor population of non-cytopathogenic viruses which can lead to persistent infection. Sequence analysis of provirus genomes recovered from the persistent infections derived from passaged virus revealed truncation of the *vpr* gene caused by the appearance of a novel stop codon as a regular mutation, and to a lesser extent, by other heterogeneous mutations [17]. These data suggested that the generation of persistent infection may be linked with infection by HIV-1 carrying, at least, the naturally occurring *vpr* nonsense mutation. Using a specific PCR procedure, we report here the increased frequency of the *vpr* mutation in a HIV-1 population during serial passage in our in vitro cell system. In addition, the *vpr* mutation was detected at a high frequency in PBMC from HIV-1 carriers confirming a link between the *vpr* truncation and persistent infection during virus transmission in nature.

2. Materials and methods

2.1. Cells and viruses

An MT-4 derived subclone M10 [8] was maintained in RPMI-1640 supplemented with 10% fetal bovine serum (complete medium). The wild-type HIV-1 was obtained from SW480 transfected with infectious molecular clone pNL432 [19].

2.2. Preparation of serial passage of HIV-1

Serial passages of HIV-1 in M10 cells up to 50 passages, designated as wt-1 to wt-50, were prepared as described previously [17]. Persistently infected M10 cells generated by the serial passaged virus were also prepared as described previously [17]. Cell clones were isolated from the M10 cells persistently infected with passage 50 by limiting dilution in a 96-well microplate as described previously [15].

2.3. Isolation of PBMC from HIV-1-seropositive subjects

The PBMC fraction from HIV-1-seropositive subjects was prepared by centrifugation on Ficoll-Hypaque (Pharmacia-LKB).

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Abbreviations: HIV-1, human immunodeficiency virus type 1; AIDS, acquired immune deficiency syndrome; PCR, polymerase chain reaction; PBMC, peripheral blood lymphocytes; SIV, simian immunodeficiency virus.

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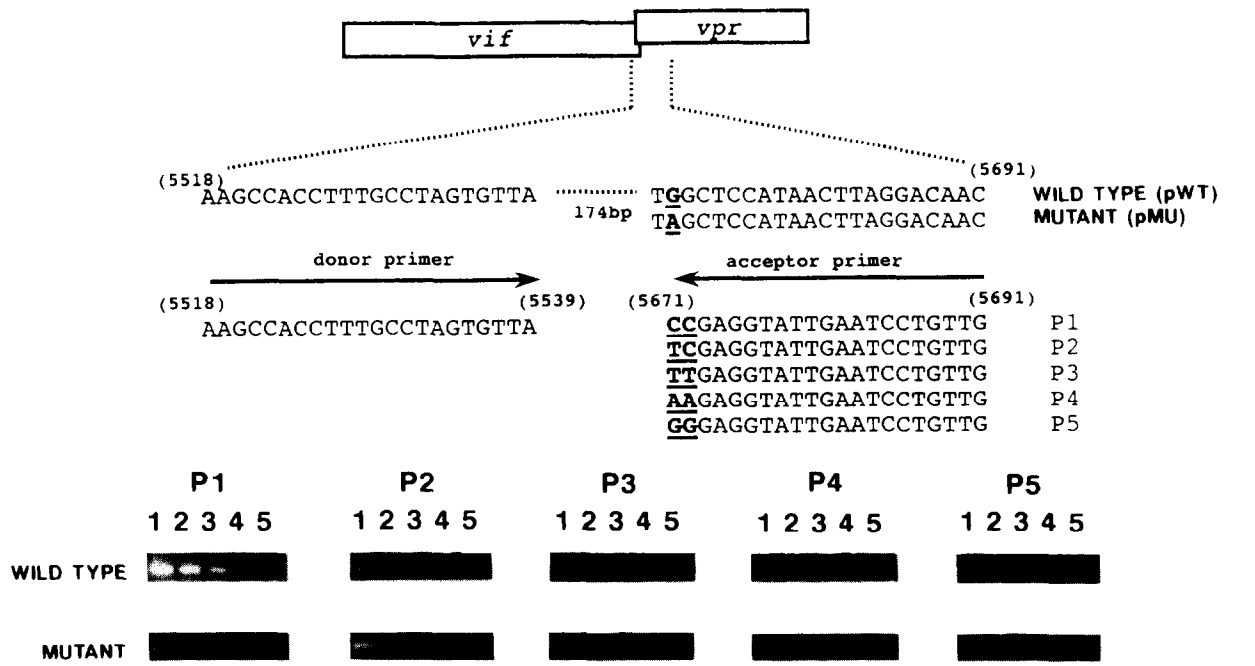


Fig. 1. Selective PCR amplification of nonsense *vpr* gene. Primer design for specific amplification of the *vpr* with nonsense mutation is shown on the top. Serial ten-fold dilutions of 1 μg pWT containing wild-type *vpr* sequence and 1 μg pMU containing *vpr* with nonsense mutation were subjected to PCR by using five acceptor primers P1 to P5 and the same donor primer as described in section 2 (bottom). The numbers 1 to 5 on the top of figures indicate 10^{-5} to 10^{-9} μg of plasmid DNA were subjected to PCR.

2.4. Semi-quantitative PCR analysis of HIV-1 genome

Total cellular DNAs were extracted from acutely or persistently HIV-1-infected cells and HIV-1 carrier-derived PBMC as described previously [15]. The amplification of extracted DNAs was performed with Taq DNA polymerase (Biotech International Ltd., Australia) in the Perkin-Elmer Cetus automated DNA thermal cycler. Each cycle consisted of denaturation at 94°C for 1 min, annealing of primers at 60°C for 2 min, and extension at 72°C for 2 min. After 30 cycles, the amplified DNA was analyzed on a 2% agarose gel, then stained with ethidium bromide.

2.5. PCR primers

For the amplification of wild-type *vpr* gene, AAGCCACCTTTGCCTAGTGTTA at nucleotides 5518 to 5539 as a donor primer and GTTGTCCTAAGTTATGGAGCC (P1) at nucleotides 5691 to 5671 as an acceptor primer were used. For the rapid assessment of the nonsense mutation in *vpr*, four other acceptor primers (P2 to P4) were examined. The underlined two bases in P1 were changed to 'CT' in P2, 'TT' in P3, 'AA' in P4, and 'GG' in P5 (Fig. 1). For the amplification of the *vpr* gene in the PBMC from HIV-1 carriers, nested PCR was also performed by using GACATAAAAGTAGTGCCAAGAAGAA at nucleotides 4995 to 5019 as donor primer and TACTTACTGCTTTGATGAGA at nucleotides 6051 to 6031 as acceptor primer for 1st PCR, followed by 2nd PCR as described above. As a control, the *gag* region of HIV-1 in PBMC from HIV-1 carriers was also amplified. The amplification of the *gag* gene was performed by nested PCR using primer sets (for 1st PCR, TTTGACTAGCGGAGGCTAGAAG at nucleotides 761 to 782 as donor primer and TTTGGTCCTTGTCTTATGTCCAGAATGC at nucleotides 1658 to 1631 as acceptor primer; and for 2nd PCR, AACAGCCAGGTCAGCCAAAATTA at nucleotides 1162 to 1184 as donor primer and ATTTAATCCCAGGATTATCCATC at nucleotides 1602 to 1580 as acceptor primer).

3. Results

3.1. Specific detection of nonsense mutation in vpr *by PCR*

The appearance of a novel stop codon TAG in place of TGG at nucleotides 5670 to 5672 in *vpr* gene of HIV-1 provirus in cells persistently infected with passaged HIV-1 was previously reported [17]. In order to quantify the mutant *vpr* sequence in infected cell populations, a PCR method to amplify only the sequence containing the stop codon was developed.

Five possible acceptor primers were first examined with the same donor primer as described in section 2. Primers P1 and P2 are matched to the wild-type and stop codon sequences, respectively, while the remaining three primers are mismatched with both sequences. A DNA clone (pWT) containing the *vif* to *vpr* region including the TGG wild-type sequence was isolated from pNL432, while a DNA clone (pMU) containing the *vif* to *vpr* sequence with the TAG stop codon was isolated from the cells persistently infected with wt-50, as described previously [17]. Serial 10-fold dilutions of these plasmids (10^{-5} to 10^{-9} μg) were subjected to PCR. Primer P1 was found to amplify a PCR product of 174 bp from both pWT and pMU (Fig. 1). However, the efficiency of amplification was different. A positive PCR reaction required one copy of pWT, while 10 copies of pMU. In contrast, the reciprocal result was obtained with primer P2. A positive PCR reaction required 10 copies of pWT but only one copy of pMU (Fig. 1). Thus, use of these different primers allows a differential detection of mutant and wild-type sequences dependent on the number of copies present in the template DNA. The P3 primer on the other hand, could amplify only the pMU not the pWT sequences. However, like P1, amplification required 10 copies of the pMU sequence. Primers P4 and P5 did not amplify product from pWT or pMU. Using this system, a semi-quantitative characterization of the proportion of wild-type TGG and mutant TAG sequences in a population was possible by using the primers P1 and P3. Next, we examined the efficiency of detection of the mutant *vpr* sequence in mixed samples with wild-type *vpr* by these two primers.

Serial tenfold dilutions, and twofold dilutions if necessary,

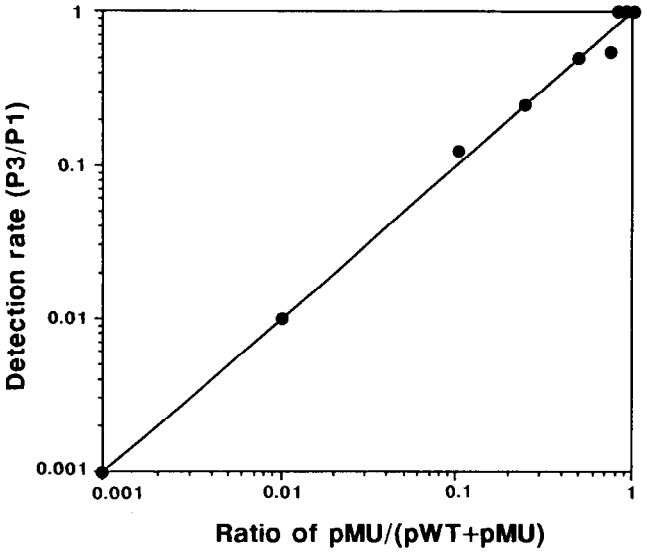


Fig. 2. Efficiency of detection of the *vpr* sequence with nonsense mutation in the mixed samples with wild-type *vpr* by PCR using P1 and P3 primers. Serial tenfold dilutions, and twofold dilutions if necessary, of 1 μg DNA samples containing pWT and pMU, in which ratios containing pMU were 0.001, 0.01, 0.1, 0.25, 0.5, 0.75, 0.9, 0.99, and 0.999, were similarly subjected to PCR using P1 or P3 acceptor primer under the same donor primer as described in the legend to Fig. 1. After electrophoresis of the PCR product, the detection rate of the DNA band by P3 to that by P1 was calculated by the highest dilution showing specific amplification.

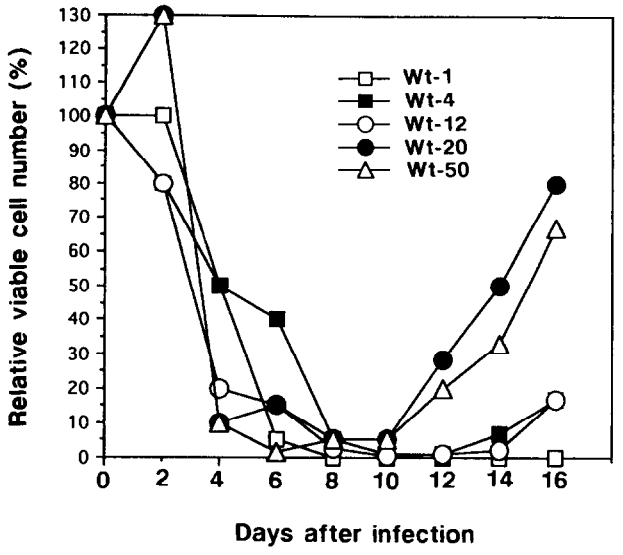


Fig. 3. Kinetics of cytopathogenic properties of serial passages of wild-type HIV-1. M10 cells were mock-infected or infected with wt-1, wt-4, wt-12, wt-20, and wt-50 at the same multiplicity of infection, 0.01. The cells were seeded at 5×10^5/ml. The media were replaced with fresh medium 4 days after infection and thereafter every 3 days. The viable cells were counted 2 and 4 days after infection and thereafter every 3 days by Trypan blue dye exclusion method. The results were shown by relative percentages of viable cell number of each infected cells, compared with that of mock-infected cells.

of 1 μg DNA samples containing different ratio of pMU and pWT (0.001 to 0.999) were similarly subjected to PCR analyses using P1 and P3 primers. The relationship between the level of *vpr* mutant sequence present in mixed samples and the detection rate by P3 and P1 is shown in Fig. 2. The relative ratio of wild-type to mutant *vpr* sequence was calculated from the regression line drawn from the data to derive the formula: $\log Y = 0.9888 \log X - 0.01197$ ($r = 0.9976$).

These data showed that the use of differential PCR could determine the level of the *vpr* mutant sequence in a mixed population of sequences and the procedure was applied subsequently to both in vitro and in vivo samples of DNA containing HIV-1.

Table 1
Ratio containing HIV-1 genome with the mutant *vpr* sequence in M10 cells acutely and persistently infected with serial passages

HIV-1 inoculum	Ratio (%) containing mutant *vpr* sequence in the cells at	
	Acute phase[a]	Persistent phase[b]
wt-1	<1	NA[c]
wt-4	0.1	1.0
wt-12	0.4	10.6
wt-20	13.2	100
wt-50	13.2	100

[a]Serial twofold dilutions of the DNAs extracted from the cells acutely infected by serial passage were subjected to PCR with primers P1 and P3. The results were calculated as percentage containing mutant *vpr* sequence compared with wild-type *vpr* sequence according to the formula from Fig. 2.
[b]Serial tenfold dilutions of the DNAs extracted from the persistently infected cells obtained after acute infection, were similarly subjected to PCR. The results were also similarly shown as for acute infection.
[c]Persistently infected cells by infection with wt-1 were not available.

3.2. *Increasing of HIV-1* vpr *mutant with serial passage*

Serial twofold dilutions of the DNAs extracted from cells acutely infected with different passages of HIV-1 were subjected to PCR amplification with the primers P1 and P3 under conditions established above. In addition, serial tenfold dilutions of the DNAs from the cells persistently infected with the passaged virus were subjected to similar PCR amplification. The results are summarized in Table 1 as the percentage of the total population containing HIV-1 genomes carrying the *vpr* nonsense mutation calculated as described for Fig. 2. In agreement with our earlier findings [17], the DNAs from the cells acutely infected with wt-1 did not amplify with P3 (<2^0) under conditions in which the same DNAs amplified up to 2^{10} dilution with P1. On the other hand, the ability to be amplified with P3 increased gradually with the passage number of the infecting viral stock, i.e. the ratios of the DNA dilution showing specific amplification with P1 to that with P3 were $2^0/2^{10}$ in wt-4, $2^2/2^{10}$ in wt-12, and $2^9/2^{12}$ in wt-20, and $2^9/2^{12}$ in wt-50. The percentages of genomes containing mutant *vpr* sequences in these acutely infected cell DNAs were calculated as 0.1%, 0.4%, 13.2%, and 13.2%, respectively. These percentages almost directly paralleled the rate of generation of persistently infected survivor cells following infection with the differently passaged viral stocks (Fig. 3). These results strongly suggest that it is the cells infected by the minor HIV-1 population carrying the *vpr* termination sequence that can survive and become persistently infected. In fact, PCR results from the DNAs derived from persistently infected cells obtained after infection with wt-20 and wt-50 showed the equivalent amplification rates with either

the P1 or P3 primers, indicating that almost 100% of the HIV-1 provirus in these cells carry the mutant form of *vpr* (Table 1). Similarly, all 15 cell clones which were isolated from persistently infected cells that were obtained after infection with wt-50 and were producers of infectious, but non-cytopathogenic, HIV-1 as described previously [17], also showed the same PCR results (not shown). However, the PCR result from the DNAs isolated from persistently wt-4- and wt-12-infected cells showed that only 1% and 10.6% of the population respectively, were the mutant form of *vpr* (Table 1).

3.3. *High rate of HIV-1* vpr *mutant in PBMC from HIV-1 carriers*

PCR amplification with primers P1 and P3 was able to identify the ratio of nonsense to wild-type *vpr* sequence (Table 1) using the formula obtained by regression line from Fig. 2. This result prompted us to apply the same procedure to detect the relative proportion of the *vpr* nonsense mutation in PBMC from HIV-1 carriers. The DNA samples from 67 PBMC of AC and 14 PBMC of ARC/AIDS patients were serially ten-fold diluted, then subjected to the PCR amplification as described for Table 1. To control for amplifiable sequences in these samples, amplification of a *gag* gene fragment was first performed using the nested PCR described in section 2. A total of 60/67 and 14/14 PBMC samples from AC and ARC/AIDS patients, respectively, were positive for the amplification of *gag* gene in undiluted total cellular DNAs (1 μg). These *gag* positive PBMC preparations were subsequently tested by the differential *vpr* PCR procedure. For these samples efficient amplification required nested PCR using the primer sets for 1st round PCR as described in section 2, followed by a 2nd round PCR using primers P1 and P3 as for Table 1.

Table 2
Detection of HIV-1 *vpr* nonsense mutation in PBMC from HIV-1 carriers

HIV-1 carrier	Clinical stage	Date for PBMC preparation	*gag*	*vpr*[a]	*vpr* stop codon[b]	HIV-1 carrier	Clinical stage	Date for PBMC preparation	*gag*	*vpr*[a]	*vpr* stop codon[b]
K.T.	AC	91.12. 9	+++[c]	+++	+++	T.H.	AC	91. 2. 4	++	+++	−
		92. 6. 1	+++	++	+			91. 4. 8	+++	++	−
T.N.	AC	91. 2. 4	+++	++	+			91. 8.12	++	++	−
		91.12. 9	+++	+++	++	O.K.	AC	91. 3. 4	+++	++++	−
		92. 6.29	+++	++	++			92. 7. 6	+++	+++	−
K.R.	AC	90.12.10	+++	+++	++	I.T.	AC	91. 4. 8	++	+++	−
N.H.	AC	93. 3. 8	+++	++	++			91. 8.12	++	++++	−
M.C.N.	AC	91. 5.27	+++	++	+	F.Y.	AC	93. 4.26	+++	+++	−
S.O.	AC	91. 6.17	+++	++	+	G.Y.	AC	91. 3. 4	++	++	−
J.D.	AC	91.12. 9	+++	+++	++			92. 1. 6	+++	++	−
N.K.	AC	91. 4.15	+++	++	+	K.M.	AC	93. 4.12	+++	+	−
						K.M.	AC	90.12.10	++	++	−
T.M.	AC	91. 1. 7	++	+++	−	T.K.	AC	91. 1.28	+++	++	−
		91. 3. 4	+++	++	++	N.H.	AC	91. 2. 4	+++	++	−
		91. 5.13	+++	+++	++	F.D.	AC	91. 1.15	+++	++	−
		91. 8.19	+++	+	−	T.N.	AC	92. 2.24	+++	++	−
		92. 5.25	++	++	−	Y.S.	AC	92. 4. 6	+++	++	−
M.H.	AC	91. 1. 7	++++	++++	++++	F.M.	AC	92. 5.25	+++	++	−
		91 3. 4	++	+++	−	H.K.	AC	93. 2. 1	+++	++	−
		91. 8.19	+	++	−	F.S.	AC	93. 3. 8	++	+++	−
		93. 2. 8	+++	++	−	T.T.	AC	93. 3.15	+++	++	−
B.N.	AC	91. 1.28	+++	++	−						
		91. 2. 4	++	++	++	N.K.	AIDS	91. 2. 4	+++	++	++
S.Y.	AC	91. 1.28	+++	++	−			92. 6. 3	+++	++	++
		92. 1.27	++	++	−	T.T.	AIDS	91. 1. 7	++	+++	++
		92. 6. 8	++	++	+	S.M.	AIDS	91. 7. 8	+++	+++	++
W.S.	AC	91. 3.25	+++	++	−						
		91. 9.30	+++	++	−	F.T.	ARC	91. 2. 4	+++	++	−
		92. 6.15	+++	++	+			91. 6.10	++	++	+
H.J.	AC	91. 5.10	++	++	−			91. 8. 5	+++	++	−
		91. 7. 8	+++	++	−	R.B.	ARC	91. 3.25	+++	+++	−
		92. 6.29	++	+++	+			91. 4. 8	+++	++	−
T.Y.	AC	91. 9. 2	+++	++	−			91. 5.27	++++	++++	+++
		93. 3.22	++	++	+						
G.M.	AC	92. 6. 8	+++	++	−	T.G.	AIDS	92. 7.13	++	++	−
		92. 6.15	+++	++	+			93. 4. 5	+++	++	−
A.N.	AC	93. 2. 1	+++	++	+	U.M.	ARC	91. 8.26	+++	+	−
		93. 3.29	++	++	−	M.S.	ARC	92. 5.25	+++	++	−
U.Y.	AC	91. 1. 7	++	++	−						
		91. 9.30	++	++	−						

[a]PCR amplification with P1 primer.
[b]PCR amplification with P3 primer.
[c]Serial 10-fold dilutions of DNA preparation (1 μg) from AC and ARC/AIDS patients were subjected to PCR amplification with *gag* primers, *vpr* primers for wild-type and mutant sequences (P1) and *vpr* primers for only mutant sequence (P3). The results were shown as follows by the highest dilution showing specific amplification: −, <1 μg; +, 1 μg; ++, 100 ng; +++, 10 ng; ++++, 1 ng.

The results (Table 2) showed that all samples were positive for amplification with primer P1, while 35.0% (21/60) and 42.9% (6/14) of the PBMC from AC and ARC/AIDS patients, respectively, were also positive for amplification with P3 primer. Thus, the proportion of mutant *vpr* sequences in the DNA samples tested was a significant level of the total population in both AC and ARC/AIDS samples. Sequential analyses of the level of the *vpr* mutant form from the same carriers showed dynamic changes in the HIV-1 population carrying the nonsense sequence in *vpr*. Over the period of the study (about 2 years and 5 months), the frequency of detection of the *vpr* mutation rose to 50% (17/34) in AC and 5/8 (62.5%) in ARC/AIDS patients.

4. Discussion

We showed previously a correlation between the ability of HIV-1 to initiate persistent infections and the occurrence of nonsense mutations in the *vpr* gene [17]. In particular, the frequency of a novel stop codon in *vpr* was revealed during a kinetic analysis of infection with in vitro serial passages of cytopathogenic HIV-1 [17]. In this communication, it was shown that the HIV-1 populations taken from acute infection contained the same mutation and that its frequency also increased progressively with serial passage (Table 1). In fact, almost 100% of the persistent HIV-1 genomes generated after infection with wt-20 and wt-50 passages were this mutant form of *vpr* (Table 1). In contrast, only 1% and 10.6% of the persistent HIV-1 genomes generated after infection with wt-4 and wt-12, respectively, contained the mutant form (Table 1). Thus, the mechanism by which persistent infections arise appears to be heterogeneous when the infecting viral stock has a low passage history. However, HIV-1 populations carrying the *vpr* nonsense mutation gradually increased with serial passage to become the predominant form of persisting virus, with persistence caused by other mutant forms present in very few genomes. A similar phenomenon in which mixed variants fail to dominate a culture during further passages has been also observed for vesicular stomatitis virus [20]. Consequently, it is likely that the Vpr truncation we have observed arises naturally during serial passage of cytopathogenic HIV-1 and is one of the key determinants for the loss of cell killing that leads to the generation of a persistent infection (Fig. 3). This result was obtained by using a specific PCR technique that was able to differentiate between the wild-type and mutant *vpr* forms by using specific *vpr* acceptor primers (Figs. 1 and 2). Under the same PCR conditions, it was found that a significant amount of HIV-1 with the same *vpr* nonsense mutation was identified in 50% and 62.5% of the wild-type *vpr* sequence-positive AC and ARC/AIDS patients, respectively (Table 2). This result supports the hypothesis that a significant population of HIV-1-seropositive individuals carry HIV-1 with the *vpr* mutation which might be a marker for the population of HIV-1 in the persistent state. In addition, sequential analyses showed a varying amount of the same nonsense *vpr* sequence in the same individual (Table 2) over time, indicating an extraordinarily dynamic flux in the *vpr* gene structure of persistent HIV-1. In fact, in the three among five rhesus monkeys infected with *vpr* mutant (conversion from the ATG start codon of the *vpr* gene to TTG) of SIV, similar reversion of the TTG sequence to ATG was also observed 4 to 8 weeks post-infection, while no *vpr* revertants were found over the entire 66 weeks of observation in the other two animals which did not develop progressive disease [21].

Investigations of the accessory genes of HIV-1 have been limited, partly due to the difficulties of readily measuring their functions by method using simple reporter gene systems such as has been possible for *tat* and *rev* [22,23]. Mutational analysis of HIV-1 *vpr* has shown that mutant grew significantly more slowly than wild-type HIV-1 [24]. In addition, *vpr* mutation of pNL432 was shown to decrease the killing of MT-4 [14,16] and H9 [25] cells. However, the molecular mechanism of this gene product is not clear at present. Recently, it was shown that the p6 region in Gag precursor protein is required for incorporation of Vpr protein into HIV-1 virions [26,27]. Subcellular fractionation studies have also suggested that a nuclear localization domain, present in Vpr, may aid transport of the viral preintegration complex to the nucleus as deletion of the carboxyl-terminal 19 amino acid arginine-rich sequence impaired Vpr nuclear localization [28]. Our model cell system which permits the generation of persistently infected cells after infection with *vpr* mutant [17] may be particularly useful for understanding the exact molecular mechanisms of the *vpr* gene product.

Analysis of the *vpr* sequence present in replication-competent HIV-1 populations in the PBMC from HIV-1 carriers is necessary to discriminate the persistent from latent forms of HIV-1. In addition, a sequential study on the *vpr* sequences present in PBMC during progression from AC to AIDS is required for understanding the possible evolution from accumulated HIV-1 with nonsense *vpr* mutation showing non-cytopathogenicity to wild-type HIV-1 showing cytopathogenic potential.

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References

[1] Temin, H.M. (1989) in: Mobile DNA (Notkins, A.L. and Oldstone, M.B.A., Eds.) American Society for Microbiology, Washington, DC.
[2] Varmus, H. and Brown, P. (1989) in: Mobile DNA (Berg, D.E. and Howe, M.M., Eds.) American Society for Microbiology, Washington, DC.
[3] Coffin, J.M. (1992) Curr. Top. Microbiol. Immunol. 176, 143–164.
[4] Benn, S., Rutledge, R., Folks, T., Gold, J., Baker, L., McCormick, J., Feorino, P., Piot, P., Quinn, T. and Martin, M. (1985) Science 230, 1548–1553.
[5] Coffin, J.M. (1986) Cell 46, 1–4.
[6] Hahn, B.H., Shaw, G.M., Taylor, M.E., Redfield, R.R., Markham, P.D., Salahuddin, S.Z., Wong-Staal, F., Gallo, R.C., Parks, E.S. and Parks, W.P. (1986) Science 232, 1548–1553.
[7] Meyerhans, A., Cheynier, R., Albert, J., Seth, M., Kwok, S., Sninsky, J., Morfeldt-Manson, L., Asjo, B. and Wain-Hobson, S. (1989) Cell 58, 901–910.
[8] Saag, M.S., Hahn, B.H., Gibbons, J., Li, Y., Parks, E.S., Parks, W.P. and Shaw, G.M. (1988) Nature 334, 440–444.
[9] Wong-Staal, F., Shaw, G.M., Hahn, B.H., Salahuddin, S.Z., Popovic, M., Markham, P., Redfield, R. and Gallo, R.C. (1985) Science 229, 759–762.

[10] Popovic, M., Sarngadharan, M.G., Read, E. and Gallo, R.C. (1984) Science 224, 497–500.
[11] Simmonds, P., Balfe, P., Peutherer, J.F., Ludlam, C.A., Bishop, J.O. and Brown, A.J.L. (1990) J. Virol. 64, 864–872.
[12] Fenyo, E.M., Morfeldt-Manson, L., Chiodi, F., Lind, B., von Gegerfelt, A., Albert, J., Olausson, E. and Asjo, B. (1988) J. Virol. 62, 4414–4419.
[13] Tersmette, M., de Goede, R.E.Y., Al, B.J.M., Winkel, I.N., Gruters, R.A., Cuypers, H.T., Huisman, H.G. and Miedema, F. (1988) J. Virol. 62, 2026–2032.
[14] Nishino, Y., Kishi, M., Sumiya, M., Ogawa, K., Adachi, A., Maotani-Imai, K., Kato, S., Hirai, K. and Ikuta, K. (1991) Arch. Virol. 120, 181–192.
[15] Kishi, M., Nishino, Y., Sumiya, M., Ohki, K., Kimura, T., Goto, T., Nakai, M., Kakinuma, M. and Ikuta, K. (1992) J. Gen. Virol. 73, 77–87.
[16] Kishi, M., Nishino, Y., Ohki, K., Kimura, T. and Ikuta, K. (1993) Jpn. J. Cancer Res. 84, 153–162.
[17] Nishino, Y., Nakaya, T., Fujinaga, K., Kishi, M., Azuma, I. and Ikuta, K. (1994) J. Gen. Virol. 75, 2241–2251.
[18] Yunoki, M., Maotani-Imai, K., Kusuda, H., Motoyama, M., Miyake, S., Imai, H., Shin, Y.S., Kato, S., Sano, K., Morita, C., Nakai, M., Hirai, K. and Ikuta, K. (1991) Arch. Virol. 116, 143–158.
[19] Adachi, A., Gendelman, H.E., Koenig, S., Folks, T., Willey, R., Rabson, A. and Martin, M.A. (1986) J. Virol. 59, 284–291.
[20] de la Torre, J.C. and Holland, J.J. (1990) J. Virol. 64, 6278–6281.
[21] Lang, S.M., Weeger, M., Stahl-Hennig, C., Coulibaly, C., Hunsmann, G., Muller, J., Muller-Hermelink, H., Fachs, D., Wachter, H., Daniel, M.M., Desrosiers, R.C. and Fleckenstein, B. (1993) J. Virol. 67, 902–912.
[22] Dayton, A.I., Sodroski, J.G., Rosen, C.A., Goh, W.C. and Haseltine, W.A. (1986) Cell 44, 941–947.
[23] Fisher, A.G., Feinberg, M.B., Josephs, S.F., Harper, M.E., Marselle, L.M., Reyes, G., Gonda, M.A., Aldovini, A., Debouk, C., Gallo, R.C. and Wong-Staal, F. (1986) Nature 320, 367–371.
[24] Ogawa, K., Shibata, R., Kiyomasu, T., Higuchi, I., Kishida, Y., Ishimoto, A. and Adachi, A. (1989) J. Virol. 63, 4110–4114.
[25] Mustafa, F. and Robinson, H.L. (1993) J. Virol. 67, 6909–6915.
[26] Paxton, W., Connor, R.I. and Landau, N.R. (1993) J. Virol. 67, 7229–7237.
[27] Lavallee, C., Yao, X.J., Ladha, A., Gottlinger, H., Haseltine, W.A. and Cohen, E.A. (1994) J. Virol. 68, 1926–1934.
[28] Lu, Y.-L., Spearman, P. and Ratner, L. (1993) J. Virol. 67, 6542–6550.