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Rapid Assessment of Phenotypic Resistance to Protease Inhibitors in Human Immunodeficiency Virus Type 1 Group O

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A bacteriophage lambda-based method was used to investigate the development of resistance to protease inhibitors (PI) in one subject infected with human immunodeficiency virus (HIV) type 1 group O who underwent multiple treatment regimens over a period of 4 years. A reduction in the susceptibility to indinavir of 6-fold and a reduction in the susceptibility to saquinavir of 24-fold were recognized after long exposure to these drugs with respect to baseline. The emergence of PI resistance corresponded to the selection of amino acid changes L10V, G48M, F53L, I54V, and L90M at the protease. The results were concordant with those obtained by a drug susceptibility assay with primary HIV isolates.

Human immunodeficiency virus type 1 (HIV-1) has been classified into three different groups, major (M), outlier (O) and non-M non-O (N) (13). Infections with HIV-1 group O are endemic in West-Central Africa, and have sporadically been reported in Europe and the United States (1, 6, 7). Like HIV-1 group M, group O may lead to AIDS in infected carriers, and therefore patients harboring this less common strain are equally eligible for antiretroviral therapy (10). Protease inhibitors (PI) along with nucleoside reverse transcriptase inhibitors are the drugs of choice, given that nonnucleoside reverse transcriptase inhibitors do not work against HIV-1 group O (5, 11, 12; D. Descamps, G. Collin, I. Loussert-Ajaka, S. Saragosti, F. Simon, and F. Brun-Vézinet, Letter, AIDS **9:**977-978, 1995). As expected, the emergence of viral variants resistant to antiretroviral drugs has been noticed in patients infected with HIV-1 group O (3, 10, 11), although knowledge on the antiviral effect of drugs against HIV-1 group O viruses is still poor. The lack of reliable methods to measure HIV-1 group O plasma viremia until recently has represented a major challenge for adequate treatment monitoring of these individuals (3, 4).

The examination of phenotypic resistance to PI when testing non-B HIV strains often requires classical cell culture procedures, which are time-consuming and difficult to perform. Recombinant virus assays have been optimized for testing phenotypic resistance in subtype B variants, but performance in testing non-B viruses is not well known. In this study, a recently described bacteriophage lambda-based method (16) was adapted to investigate the proteolytic activity of HIV-1 group O proteases and therefore to assess their susceptibilities to specific inhibitors. This method is rapid, safe, and easy to perform. Results show that susceptibility measurement was similar to that by the phage-based assay and a more standard virus isolate-based methodology.

Clinical samples. Plasma specimens were collected from one individual infected with HIV-1 group O who began attending our institution in 1995. The patient (ESP2) was a 35-year-old Spanish woman known to be HIV positive since 1995, when she developed *Pneumocystis carinii* pneumonia (6). She began antiretroviral therapy at that time, and since then she has been exposed to multiple regimens, including both nucleoside reverse transcriptase inhibitors and PI.

The patient most likely had acquired HIV infection through heterosexual relationships with her husband (ESP1), a 36-year-old Spanish male, who had been working in Equatorial Guinea and Cameroon during the previous years (6). Plasma specimens collected longitudinally from this individual, who has been without antiretroviral therapy so far, were used for comparison studies in cell culture phenotypic assays. The main features of specimens from these two patients are summarized in Table 1. Plasma viremia was measured using the recently described LCx technology, which provides reliable values for HIV-1 group O (4).

Construction of recombinant phages. Viral RNA was extracted from plasma using the SV total RNA isolation kit (Promega, Madison, Wis.). The HIV-1 group O protease gene was amplified by reverse transcription (RT)-PCR (Titan kit; Roche Molecular Biochemicals, Mannheim, Germany), followed by nested PCR using 5′protO (5′ GCAAATTTTTTAG GCAAATACTGGCCTCC 3′) and 3′protO (5′ AAAGATAG GTGTATTATAAGGATTTTCAGG 3′) as outer primers and HIVOprotEc (5′ GGGGAATTCCGAGGCCAGGCAATT 3′) and HIVOproXH (5′ GGGAGGGGCTCGAGTCAAAG TCCATCCATTCCTGG 3′) as inner primers. Briefly, the RT-PCR was performed following the manufacturer's instructions under the following conditions: 50°C incubation for 30 min, followed by one denaturation cycle at 94°C for 2 min. Then, 10 cycles of 94°C for 30 s, 55°C for 30 s, and 68°C for 1 min were carried out. Thereafter, 25 cycles of 94°C for 30 s, 55°C for 30 s, and 68°C for 1 min, plus 5 s per cycle, were carried out. A final extension step at 68°C for 7 min was performed. Then, 5 μl of the RT-PCR product was amplified using the following cycling conditions: 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, with a final incubation step of 72°C for 7 min. The amplicon flanked by *Eco*RI and *Xho*I restriction sites was cloned into an *Eco*RI/*Xho*I-predigested lambda phage expression vector (Uni-ZAP, XR vector and cloning kit; Stratagene, La Jolla, Calif.) and used to trans-

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TABLE 1. Main features of specimens collected from two patients infected with HIV-1 group O

Specimen	Collection date	Treatment regimen (duration)	CD4 count (cells/μl)	Plasma viremia (HIV RNA copies/ml)
ESP2-0	April 1995	None	105	18,756
ESP2-1	February 1997	Stavudine, lamivudine, indinavir (4 mo)	165	9,084
ESP2-2	January 1998	Stavudine, lamivudine, saquinavir, ritonavir (7 mo)	143	48,798
ESP2-3	February 1999	Stavudine, didanosine, nelfinavir (10 mo)	99	61,905
ESP2-4	May 1999	Zidovudine, lamivudine, indinavir, ritonavir (11 mo)	121	98,356
ESP1-0	April 1995	None	290	ND[a]
ESP1-1	January 1998	None	125	4,004

[a] ND, not determined.

form *Escherichia coli* to produce large amounts of the recombinant phages.

DNA sequencing analysis. HIV-1 group O protease genes from clinical specimens at different time points were sequenced using the dRhodamine dye terminator sequencing kit (Applied Biosystems, Foster City, Calif.), according to the manufacturer's instructions. Sequences were obtained using an ABI 310 genetic analyzer (Applied Biosystems), translated using Sequence Navigator software (version 1.0.1; Applied Biosystems), and aligned with the HIV-1 group O reference sequence ANT70 by using Clustal X software.

Figure 1 depicts the predicted amino acid sequences found in the study samples. Briefly, specimens collected from ESP2 before beginning antiretroviral therapy (ESP2-0) and from ESP1 at any time were highly concordant with the ANT70 reference sequence. Interestingly, all these sequences harbored L10I, M36I, and A71V changes, which may be considered to be naturally occurring polymorphisms in HIV-1 group O. They are involved in the development of PI resistance in HIV-1 subtype B (14). Whether HIV-1 group O viruses may show a reduced susceptibility to PI based on these changes is not well known (3). Our results, however, show that changes such as M36I and A71V appear in all group O viruses and do not confer resistance to indinavir and/or saquinavir, in the absence of primary PI resistance mutations (see below). This is in agreement with preliminary results obtained by Descamps et al. (5).

Primary mutations associated with PI resistance in HIV-1 subtype B (14) were recognized in specimens collected from ESP2 after beginning antiretroviral therapy. Sample ESP2-1, collected after 4 months on indinavir, showed L10V. Sample ESP2-2, collected after 7 months on saquinavir boosted with ritonavir and previous exposure to indinavir for 9 months, showed mutations G48 M, F53L, I54V, and L90 M. The same mutations were seen in specimens ESP2-3 and ESP3-4, collected much later (Table 1).

Lambda-based phenotypic assay. The phage lambda-expressing system described by Sices and Kristie (16) and used to measure drug susceptibilities in HIV-1 group M proteases by Martinez et al. (9) and Sices et al. (17), was adapted to study the HIV-1 group O protease activity. Briefly, the method is based in the *cI-cro* regulatory system, which controls the phage lysogenic cycle-lytic cycle switch. When a phage expressing the HIV-1 protease infects *E. coli* carrying a recombinant *cI* repressor containing a specific HIV-1 cleavage site, the phage goes into the lytic replication cycle. The peptide recognized by the HIV-1 protease is the p17/p24 matrix-capsid *gag* cleavage site (VSQNYPIV), which is highly conserved across HIV strains. Although HIV-1 group O viruses harbor AGQNYPIV, the protease cleavage does not seem to be altered (2). Therefore, we decided to use this system for characterizing the protease activity and the susceptibilities to PI of HIV-1 group O viruses. The method has advantages over standard cell culture assays. It can be performed in only 3 days, and it is easy and inexpensive.

E. coli JM109 cells containing pET cI.HIV and pALTER cI.HIV-cro plasmids were infected with 2×10^7 PFU of recombinant phages carrying the different HIV-1 group O proteases. Then, bacteria were washed with 1 ml of 10 mM $MgSO_4$ and incubated at 37°C in Luria-Bertani medium containing tetracycline (12.5 μg/ml), 0.2% maltose, 10 mM $MgSO_4$, 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and different concentrations of the PI under evaluation (indinavir and saquinavir at 0, 0.5, 2.5, 12.5, 62.5, and 313 μM). After 3 h, new medium was added, and infected *E. coli* was left to incubate at 37°C for another 3 h. Aliquots of cultures were afterwards coplated with *E. coli* XL-Blue in top agar containing tetracycline (12.5 μg/ml), 0.2% maltose, 0.1 mM IPTG, and the corresponding concentration of drug. Incubation at 37°C extended for 6 h. Protease activity was measured by plaque formation. Protease from the HIV-1 HXB2 strain (λ-HIV-1pHXB2) was used as a drug-sensitive control. A lambda phage carrying an

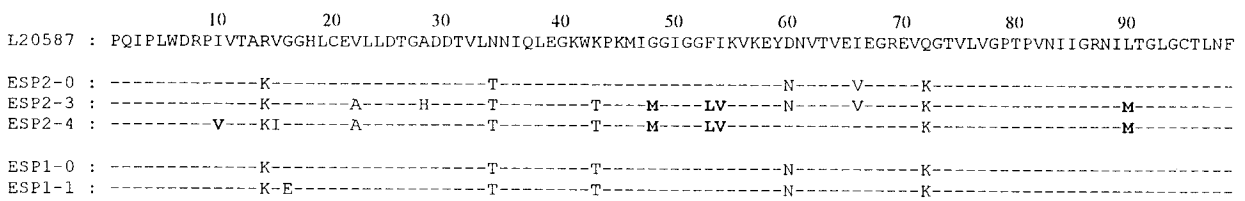

FIG. 1. Multiple amino acid sequence alignment at the protease in specimens taken at different time points from two patients infected with HIV-1 group O. The top sequence corresponds to the group O consensus amino acid. Only those amino acids differing from the consensus are given. Dashes indicate the same amino acid. Mutations associated with PI resistance are shown in boldface type.

TABLE 2. Sensitivities of different HIV-1 group O proteases to indinavir and saquinavir using the lambda-based phenotypic method and the standard in vitro drug susceptibility assay

Method	Sample (mutation pattern)	IC_{50} (nM) (fold increase) of:	
		Indinavir	Saquinavir
Lamda phage	HXB2 (WT[a])	25 ± 2	2.57 ± 0.88
	ESP2-0 (WT)	28 ± 5.75	5.08 ± 0.41
	ESP2-4 (L10V, G48M, F53L, 154V)	150 ± 32.25 (5,4)	122.67 ± 39.5 (24)
Standard cell culture assay	HXB2	ND[b]	ND
	ESP1-2 (WT)	15.13	5 ± 0.78
	ESP2-3 (L10V, G48M, F53L, 154V)	185.5 ± 53.5 (12)	1,264.7 ± 285 (>200)

[a] WT, wild type.
[b] ND, not done.

inverted HXB2 protease (λ-HIV-1pinvHXB2) was used as a negative control.

The recombinant lambda phages expressing HIV-1 group O proteases derived from longitudinal clinical samples were examined for their phenotypic susceptibilities to different PI. The mean 50% inhibitory concentration (IC_{50}) of indinavir for ESP2-0 was similar to that seen for the HXB2 control strain (28 and 25 μM, respectively), while the IC_{50} of indinavir for ESP2-4 was almost six times higher (150 μM). ESP2-4 showed a 24-fold-increased IC_{50} of saquinavir (122 μM) compared to that for ESP2-0 (5 μM) (Table 2).

The phenotypic results were examined in parallel with the genotypic data. No primary mutations in the protease gene were identified in samples collected at baseline from ESP2, nor were primary mutations identified in any of those from ESP1, who always had been without therapy. However, multiple changes at positions classically associated with PI resistance in subtype B viruses were recognized in samples collected from ESP2 after being on antiretroviral therapy. To further validate the phage lambda-based method for drug susceptibility testing, these results were compared with those obtained by the standard cell culture phenotypic assay.

Cell culture drug susceptibility assay. Primary virus isolates obtained from samples ESP1-1 and ESP2-3 were used to infect phytohemagglutinin–interleukin-2-stimulated peripheral blood mononuclear cells from HIV-seronegative blood donors with different concentrations of antiretroviral drugs (indinavir and saquinavir at 0, 0.001, 0.01, 0.1, 0.5, 1, and 5 μM). One thousand 50% tissue culture infective doses of each virus stock was used to infect 10^6 peripheral blood mononuclear cells. The effect of drug inhibition on viral replication was determined by measuring p24 antigen levels in culture supernatants 7 days after infection (Fig. 2). The IC_{50} was calculated after performing different experiments and expressed as mean ± standard deviation. Genetic sequence analyses of ESP1-1 and ESP2-3 specimens showed the same amino acid substitutions that were recognized in samples tested using the lambda-based assay. The IC_{50}s of indinavir and saquinavir for wild-type HIV-1 group O viruses were similar to those of wild-type subtype B viruses. Results given in Table 2 show that ESP2-3 had a 10-fold-reduced susceptibility to indinavir and >200-fold reduced susceptibility to saquinavir. Although some quantitative differences in saquinavir susceptibilities were found comparing the cell culture assay and the lambda phage assay (Table 2), overall they were in agreement, supporting the use of the lambda-based assay for assessing PI susceptibilities in subjects infected with diverse HIV-1 variants.

Characteristically, samples from ESP2 expressing high-level phenotypic resistance to saquinavir harbored mutations at positions 48 and 90 of the protease. These positions are typically

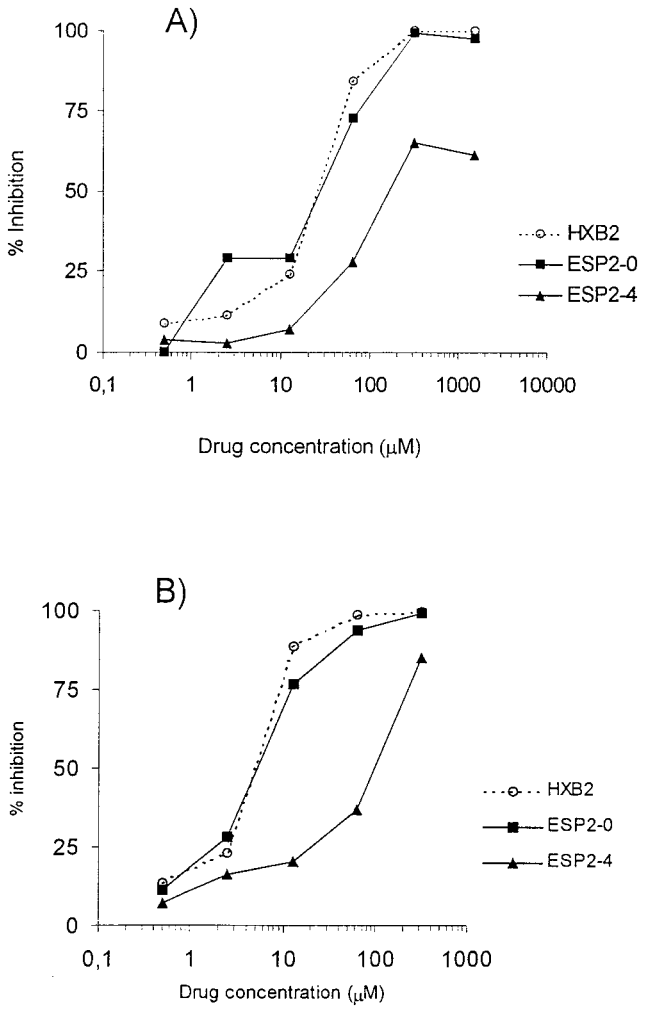

FIG. 2. Inhibition curves obtained in the lambda phage assay using indinavir (A) and saquinavir (B) to test HXB2 and clinical samples belonging to ESP2.

associated with resistance to saquinavir in subtype B viruses (14). In addition, multiple changes at other codons were found in samples collected from ESP2, which most likely contributed to the production of a moderate loss of susceptibility to indinavir. Finally, the clinical, virologic, and immunologic outcome in ESP2 was in agreement with the described genotypic and phenotypic data (Table 1).

In summary, this study showed that a rapid, simple and safe lambda phage system yielded similar drug susceptibility results as a virus isolate-based tissue culture assay for group O isolates from two subjects. The lambda phage-based method developed by others and previously used for different purposes (9, 16, 17) was adapted here to permit cloning and assay of group O proteases. The subject whose specimens contained resistant group O protease had mutations in those specimens that are common in subtype B resistant viruses (15). Naturally occurring polymorphisms in clade O did not affect susceptibility (8, 12). The concordance between the genetic assay, the standard phenotype assay, and the phage-based assay suggests that phage-based phenotyping may be adaptable to clinical applications.

Nucleotide sequence accession numbers. HIV-1 group O sequences generated in this study were given the following GenBank accession numbers: AF469050, AF469051, AF469052, AF469053, and AF469054.

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Volume 40, no. 11, p. 4313–4316, 2002. Page 4316: The Acknowledgments section was inadvertently omitted and should appear as shown below.

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