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Susceptibility of Human Immunodeficiency Virus Type 1 Group O Isolates to Antiretroviral Agents: In Vitro Phenotypic and Genotypic Analyses

DIANE DESCAMPS,[1]* GILLES COLLIN,[1] FRANCK LETOURNEUR,[2] CRISTIAN APETREI,[1,3] FLORENCE DAMOND,[1] IBTISSAM LOUSSERT-AJAKA,[1] FRANÇOIS SIMON,[1] SENTOB SARAGOSTI,[2] AND FRANÇOISE BRUN-VÉZINET[1]

Laboratoire de Virologie, Hôpital Bichat-Claude Bernard,[1] and Institut Cochin de Génétique Moleculaire, Institut National de la Santé et de la Recherche Médicale, Hôpital Cochin,[2] Paris, France, and Virus Laboratory, School of Medicine, "Gr. T. Popa" University of Iasi, Iasi, Romania[3]

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We investigated the phenotypic and genotypic susceptibility of 11 human immunodeficiency virus type 1 (HIV-1) group O strains to nucleoside and nonnucleoside reverse transcriptase (RT) inhibitors and protease inhibitors in vitro. Phenotypic susceptibility was determined by using a standardized in vitro assay of RT inhibition, taking into account the replication kinetics of each strain. HIV-1 group M and HIV-2 isolates were used as references. DNA from cocultured peripheral blood mononuclear cells was amplified by using *pol*-specific group O primers and cloned for sequencing. Group O isolates were highly sensitive to nucleoside inhibitors, but six isolates were naturally highly resistant to all of the nonnucleoside RT inhibitors tested. Phylogenetic analysis of the *pol* gene showed that these isolates formed a separate cluster within group O, and genotypic analysis revealed a tyrosine-to-cysteine substitution at residue 181. Differences in susceptibility to saquinavir and ritonavir (RTV) were not significant between group O and group M isolates, although the 50% inhibitory concentration of RTV for group O isolates was higher than that for the HIV-1 subtype B strains. The study of HIV-1 group O susceptibility to antiretroviral drugs revealed that the viruses tested had specific phenotypic characteristics contrasting with the group M phenotypic expression.

At least 10 distinct human immunodeficiency virus type 1 (HIV-1) clades or subtypes, designated subtypes A to J (19) on the basis of the *env* gene, have been identified, and each subtype is approximately equidistant from the others in phylogenetic patterns (11). This extensive variability might have a significant impact on HIV infection epidemiology (18), diagnosis (1), and treatment (3, 5) and the follow-up of HIV-1-infected patients (14).

Two viruses from Cameroonian patients (ANT70 and MVP5180) radically different from other HIV-1 sequences and clustering together have been fully sequenced and formed a new HIV-1 subtype designated O (for outlier) (8, 27). Recently, Charneau et al. (4) suggested splitting HIV-1 into two groups, M (for major) and O, with evolutionary radiation from two different ancestors (13). The circulation of HIV group O seems to be restricted to West-Central Africa, where it accounts for less than 10% cases of HIV-1 infection (20), but HIV-1 group O infection has been detected in France (15), Germany (9), Spain (24), and recently the United States (7), mainly in patients from Central Africa.

In a preliminary study in 1995 (5), we reported that HIV-1 group O isolates from eight Cameroonian patients living in France were resistant to a nonnucleoside reverse transcriptase (RT) inhibitor (NNRTI) (TIBO R82913) and susceptible to nucleoside RT inhibitors (NRTIs). The objectives of the present study were to identify drugs which can be used for the treatment of HIV-1 group O-infected patients and to investigate the molecular basis of the susceptibility or resistance of these strains. We report phenotypic and genotypic data on 11 HIV-1 group O isolates vis-a-vis three classes of antiretroviral compounds (NRTIs, NNRTIs, and protease inhibitors).

Patients. Ten HIV-1 group O-infected patients living in France (eight Cameroonian and two French) were included. HIV-1 group O infection was confirmed by *gag* and C2-V3 *env* sequence analysis (13). The VAU and RUD isolates were obtained from Pitié-Salpétrière (D. Candotti and J. M. Huraux) and the Pasteur Institute (J. Cohen and L. Montagnier), and HIV-1 group O isolate MVP5180 (8) was kindly provided by L. Gürtler from the Max von Pettenkofer-Institut für Hygiene und Medizinische Mikrobiologie. Clinical and epidemiological data of the patients in the study group, virus isolation, *gag* phylogenetic analyses, and strain codification were described elsewhere (13). Only one patient (the one with isolate BCF13) had received antiretroviral drugs (zidovudine [ZDV] at 600 mg/day for 1 year, combined with lamivudine [3TC] at 600 mg/day for 6 months) prior to the study.

Phenotypic susceptibility assay. The phenotypic susceptibility of the cellular HIV-1 group O isolates was analyzed in a peripheral blood mononuclear cell assay as previously described (2). We tested the NRTIs ZDV (Glaxo-Wellcome, Dartford, United Kingdom), didanosine (ddI; Bristol-Myers-Squibb, Wallingford, Conn.), zalcitabine (ddC; Roche, Welwyn Garden City, United Kingdom), and 3TC (Glaxo-Wellcome) and the NNRTIs TIBO derivative R82913 (Janssen, Beerse, Belgium), delavirdine (DLV; Upjohn, Kalamazoo, Mich.), and nevirapine (NVP; Boehringer Ingelheim Pharmaceuticals, Ridgefield, Conn.). Complementary studies were performed with the protease inhibitors saquinavir (SQV; Roche) and ritonavir (RTV; Abbott, Abbott Park, Ill.). Purified drugs were kindly provided by the manufacturers. Phenotypic resistance to the antiretroviral agents was defined as at least a fivefold in-

* Corresponding author. Mailing address: Laboratoire de Virologie, Hôpital Bichat-Claude Bernard, 46 rue Henri Huchard, 75018 Paris, France. Phone: 33-1-40-25-88-96. Fax: 33-1-46-27-02-08. E-mail: diane.descamps@bch-ap-hop-paris.fr

crease in the 50% inhibitory concentrations (IC_{50}s) for HIV-1 group O isolates compared to the HIV-1 subtype B (LAI) reference strain, except for ZDV, for which decreased susceptibility was defined by IC_{50}s of 0.05 μM or more.

As shown in Table 1, all but two group O isolates were susceptible to the NRTIs tested (ZDV, ddI, ddC, and 3TC). For strains MVP5180 and BCF13, the 3TC IC_{50}s were 6- and >125-fold higher than for the 3TC-susceptible HIV-1 group M (subtype B) strains, respectively.

Six group O isolates (BCF01, BCF02, BCF03, BCF07, BCF08, and BCF13) were resistant to NVP, with IC_{50}s ranging from 250-fold to 1,025-fold higher than for susceptible HIV-1 group M strains (Table 1). Two strains (BCF11 and MVP5180) showed a decrease in susceptibility to this compound, with IC_{50}s 15- and 16.5-fold higher than for susceptible strains. Three isolates (BCF06, VAU, and RUD) were considered susceptible. The same six group O isolates (BCF01, BCF02, BCF03, BCF07, BCF08, and BCF13) exhibited a strain-related difference in DLV susceptibility, with IC_{50}s ranging from 17.5- to 1,000-fold higher than for sensitive group M strains. Five isolates (VAU, BCF06, RUD, BCF11, and MVP5180) were susceptible. Nine isolates (BCF01, BCF02, BCF03, BCF06, BCF07, BCF08, BCF11, BCF13, and MVP5180) were resistant to the TIBO derivative R82913, with more than 48-fold higher IC_{50}s than for HIV-1 group M strains. One isolate (RUD) exhibited a smaller decrease in susceptibility (8.5-fold higher IC_{50}), and one isolate (VAU) was susceptible.

All of the strains tested were susceptible to SQV (Table 1). Susceptibility to the other protease inhibitor (RTV) ranged from susceptible to borderline, the ratio of IC_{50}s for group O isolates and susceptible HIV-1 subtype B strains ranging from 0.45 to 5.1.

PCR and cloning. DNA from cocultured peripheral blood mononuclear cells of infected patients was extracted with phenol-chloroform, precipitated with ethanol, and quantified spectrophotometrically. All of the primers we used (Table 2) were selected from conserved regions in the consensus sequence of HIV-1 subtype O isolates ANT70 and MVP5180 (8, 27), except for primer POL-4481, which was selected from the HIV-1 subtype B consensus sequence corresponding to the same region of the consensus O sequence. The positions of these primers correspond to nucleotides of the HIV-1 MVP5180 sequence (Table 2).

The *pol* gene was amplified in a nested PCR. The first round of PCR generated a large fragment of 3,724 bp with the XL DNA PCR kit (Perkin Elmer) used in accordance with the manufacturer's instructions with forward primer GAGCAM-EXT5′ and reverse primer POL-4481. The second round of PCR was performed with sense primer GAGCAM-INNER5′ and antisense primer POLCAM52, generating a fragment of 2,107 bp. The PCR for the nested step was carried out in 100-μl reaction mixtures containing 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM (each) deoxynucleoside triphosphate, 40 pmol of each primer, 2.5 U of *Taq* polymerase (Boehringer), and 2 μl of the amplified product of the first reaction step. Samples were submitted to 35 amplification cycles, each consisting of three successive steps: denaturation at 94°C for 30 s, annealing of primers at 55°C for 30 s, and extension at 72°C for 90 s. In the first cycle, denaturation was performed for 5 min, and in the last cycle, it was extended to 7 min. Amplified products were purified on columns with the Qiagen kit (Qiagen). Sense primer POLCAM50 and antisense primer POLCAM52 were used to amplify a fragment of 762 bp in the *pol* gene. Amplified products were digested with the *Eco*RI restriction enzyme, purified, and cloned in M13.

TABLE 1. IC_{50}s of NRTIs, NNRTIs, and protease inhibitors for HIV-1 group O isolates compared to HIV-1 group M and HIV-2 ROD isolates

Virus	Mean IC_{50} ± SD (no. of isolates)								
	ZDV (μM)	ddI (μM)	ddC (μM)	3TC (μM)	NVP (μM)	DLV (μM)	R82913 (μM)	SQV (nM)	RTV (nM)
HIV-1 LAI	<0.01	0.85 ± 0.4 (5)	0.15 ± 0.1 (5)	0.05 ± 0.03 (5)	<0.10	0.01	0.02	12.2	12
Group M isolates	<0.03 (127)	0.7 ± 0.3 (15)	0.06 ± 0.04 (6)	0.05 ± 0.05 (6)	0.04 ± 0.01 (10)	0.02 ± 0.01 (16)	0.13 ± 0.08 (10)	10.3 ± 2.5 (12)	55 ± 26 (10)
HIV-2 ROD	<0.01	0.5 ± 0.1 (5)	0.05 ± 0.03 (5)	0.02	>62.5	>20	>6.25	9.8	80
HIV-1 group O isolates									
MVP5180	<0.01	ND[a]	ND	0.31	0.66	0.12	>6.25	13	85
BCF01	<0.01	<0.125	0.02	0.02	15	1.2	>6.25	20.4	181
BCF02	<0.01	0.3	0.03	0.04	21	7.2	>6.25	13.2	25
BCF03	<0.01	1.2	0.12	0.02	41	>20	>6.25	12.3	61
VAU	0.01	0.5	ND	0.06	0.1	<0.02	0.08	13	170
BCF06	0.03	0.9	0.16	0.08	0.12	0.2	>6.25	13.8	68
BCF07	<0.01	0.5	0.06	<0.01	17	0.9	>6.25	12.5	137
BCF08	<0.01	0.2	0.06	<0.01	10	2.3	>6.25	16.4	25
RUD	<0.01	ND	ND	0.04	0.13	0.04	1.1	13	115
BCF11	0.01	0.45	0.05	0.02	0.59	0.06 ± 0.03 (3)	>6.25	22.5	43
BCF13	<0.01	1.2	0.06	>6.25	19	0.35	>6.25	11.9	283

[a] ND, not done.

TABLE 2. Oligonucleotide primers used for PCR and sequencing

Primer	Sequence	Orientation	Positions
GAGCAM-EXT5′	GAGAATTCCAGGGACAAATGGTACATCA	Sense	1225–1244
GAGCAM-INNER5′	GAGAATTCTAAATGCATGGGTAAAGGCAGT	Sense	1265–1286
GAGP7	GGTCACCAAATGAAAGAT	Sense	2086–2103
POL-4481	GCTGTCCCTGTAATAAACCCG	Antisense	4929–4949
POLCAM50	AGGAATTCGATGGACCAAAAGTAAAACAAT	Sense	2631–2649
POLCAM52	AGGAATTCGATAGATTTGACTTGCCCAAT	Sense	3373–3393
POLCAM53	ATCTTTCTTTTTTATAGCAAA	Antisense	2760–2780
POLCAM54	CCCTATCATTTTTGGTGTC	Antisense	2408–2426
POLCAM55	GGATACAGGGGCAGATGA	Sense	2354–2371

Sequencing. For each isolate, the DNA fragment generated by primers GAGCAM-INNER5′ and POLCam52 was submitted to direct population sequencing with sense primers GAGP7, POLCAM55, and POLCAM50 and antisense primers POL-CAM52, POLCAM53, and POLCAM54. Sequencing reactions were performed with the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA polymerase FS (Perkin Elmer) on an automated DNA sequencer (Applied Biosystems 373A sequencer). The *pol* gene sequences from position 2631 to position 3393 were checked on cloned products for all of the isolates except RUD. Three clones were sequenced for each patient with the PRISM Ready Reaction Ampli *Taq* dye primer-21M13 kit (Perkin Elmer).

Figure 1 depicts alignments of amino acid sequences deduced from PCR fragments. These HIV-1 group O sequences are shown in comparison with those of several HIV-1 group M

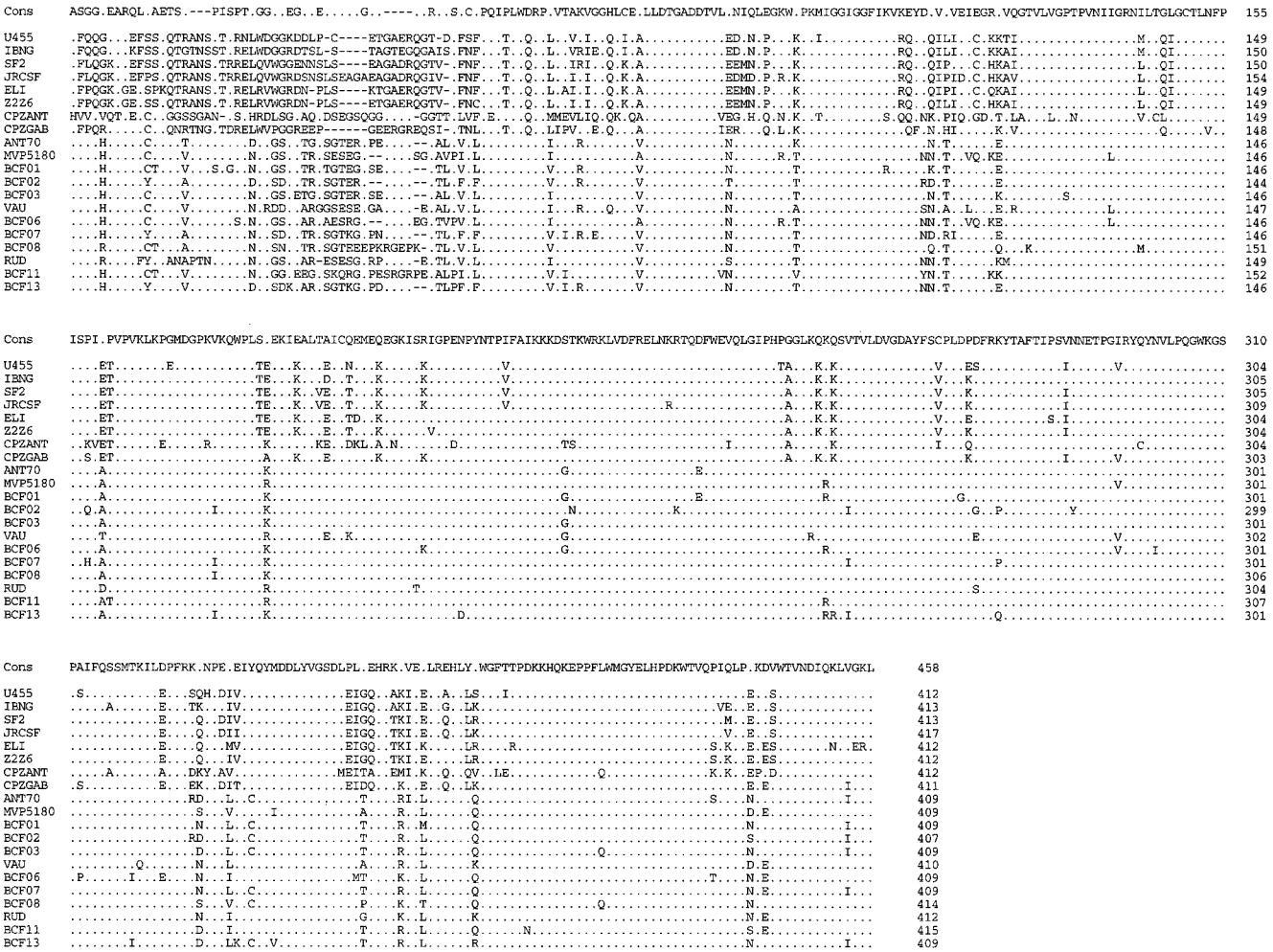

FIG. 1. Alignment of deduced amino acid sequences of the amino-terminal region of the *pol* gene, the protease gene, and a fragment of RT of group O strains, shown in comparison with HIV-1 subtypes A, B, and D of group M, as well as SIV CPZ. The top line corresponds to the consensus sequences (Cons). Due to the presence of a greater number of group O sequences, the consensus sequence corresponds to the group O consensus sequence.

TABLE 3. Substitutions in the RT gene associated with decreased susceptibility to NNRTIs in HIV-1 group M isolates

HIV-1 group O isolate	Amino acid at position indicated compared with following known substitution in group M RT gene:										Fold increase in IC_{50}[a] of:		
	98 A→G	100 L→I	103 K→N	106 V→A	108 V→I	138 E→K	181 Y→C	188 Y→H/L/C	190 G→A	236 P→L	NVP	DLV	R82913
MVP5180	G	L	R	V	V	E	Y	Y	G	P	16.5	5.4	>48
BCF01	G	L	R	V	V	E	C	Y	G	P	375	60	>48
BCF02	G	L	K	I	V	E	C	Y	G	P	525	360	>48
BCF03	G	L	K	V	V	E	C	Y	G	P	1,025	1,000	>48
VAU	G	L	K	V	V	E	Y	Y	G	P	2.5	2	1.2
BCF06	G	L	R	V	V	E	Y	Y	G	P	3	2.2	>48
BCF07	G	L	K	I	V	E	C	Y	G	P	425	45	>48
BCF08	G	L	K	V	V	E	C	Y	G	P	250	115	>48
RUD	G	L	K	V	V	E	Y	Y	G	P	3.2	2	9.2
BCF11	G	L	R	V	V	E	Y	Y	G	P	15	2.7	>48
BCF13	G	L	R	I	V	E	C	Y	G	P	475	17.5	>48

[a] The fold increase in IC_{50} for HIV-1 group O isolates corresponds to the HIV-1 group O/group M IC_{50} ratio.

and O isolates, as well as simian immunodeficiency virus (SIV) CPZ from the Los Alamos National Laboratory Database (19). The amino-terminal region of the *pol* gene, upstream of the protease gene, showed a striking divergence in terms of size and amino acid composition among the group M, group O, and SIV CPZ sequences. Viruses from group O-infected patients harbored several amino acid substitutions relative to the North American-European clade B consensus sequence, which has previously been linked to resistance to antiretroviral agents (16) (Fig. 1). Concerning NRTI susceptibility, in vitro phenotype results were confirmed by genotyping, as the two 3TC-resistant strains had an isoleucine substitution (MVP5180) and a valine substitution (BCF13) at codon 184 of the RT gene. All of the other susceptible isolates were the same as wild-type group M strains in the RT gene at codons identified as associated with resistance to NRTIs in group M (16).

For NNRTIs, we observed different patterns of substitutions (Table 3). All of the isolates sequenced harbored a glycine at position 98. Six isolates (BCF01, BCF02, BCF03, BCF07, BCF08, and BCF13) had a cysteine at codon 181. Other identified substitutions were an arginine at position 103 (BCF01, BCF06, BCF11, BCF13, and MVP5180) and an isoleucine at position 106 (BCF02, BCF07, and BCF13).

None of the isolates showed mutations associated with resistance to SQV (10), which is in agreement with the phenotypic susceptibility results. By contrast, sequence analyses showed substitutions in different codons of the protease gene which have been linked to resistance to RTV (17). Thus, we detected the L10I substitution in five isolates (BCF03, VAU, BCF06, RUD, and MVP5180), the M36I substitution in all of the HIV-1 group O isolates tested so far, and the A71V substitution in all isolates but one (RUD). However, these mutations were not associated with changes in phenotypic susceptibility. We observed no significant difference between group M and group O isolates in the active site (amino acids [aa] 21 to 32), the flap (aa 47 to 56), or the substrate binding site (aa 78 to 88) of the protease gene (23). Isolate BCF13, for which the RTV IC_{50} was the highest, did not exhibit a particular pattern of mutations in the protease gene (Table 4).

Phylogenetic analysis. DNA sequences were analyzed by using the multiple sequence editor Clustal W (26) and improved by visual inspection (Fig. 2). The sequences were gap stripped, and a pairwise matrix based on 1,182 sites was generated with the DNADIST program in the PHYLIP package, version 3.56 (6). Tree topology was inferred by the neighbor-joining method with the Clustal W bootstrap option (100 resamplings). Phylogenetic analysis was also performed by the maximum-likelihood method with the DNAML program (6). Six group M sequences representative of different subtypes, as

TABLE 4. Substitutions in the protease gene associated with decreased susceptibility to protease inhibitors SQV and RTV in HIV-1 group M isolates

HIV-1 group O isolate	Amino acid at position indicated compared with following known substitution in group M protease gene:													Fold increase in IC_{50}[a] of:	
	10 L→I	20 K→R	24 L→I	36 M→I	46 M→I	48 G→V	54 I→V	63 L→P	64 I→V	71 A→V	82 V→A/T/F	84 I→V	90 L→M	SQV	RTV
MVP5180	I	C	L	I	M	G	I	T	V	V	V	I	L	1.3	1.5
BCF01	V	C	L	I	M	G	I	T	V	V	V	I	L	2	3.2
BCF02	V	C	L	I	M	G	I	T	V	V	V	I	L	1.3	0.45
BCF03	I	C	L	I	M	G	I	T	V	V	V	I	L	1.2	1.2
VAU	I	C	L	I	M	G	I	A	V	V	V	I	L	1.3	3.1
BCF06	I	C	L	I	M	G	I	T	V	V	V	I	L	1.3	1.2
BCF07	V	C	L	I	M	G	I	R	I	V	V	I	L	1.2	2.5
BCF08	V	C	L	I	M	G	I	T	V	V	V	I	L	1.6	0.45
RUD	I	C	L	I	M	G	I	T	V	M	V	I	L	1.3	2.1
BCF11	V	C	L	I	M	G	I	T	V	V	V	I	L	2.2	0.8
BCF13	V	C	L	I	M	G	I	T	V	V	V	I	L	1.2	5.1

[a] Fold increase in IC_{50} for group O isolates compared to group M isolates.

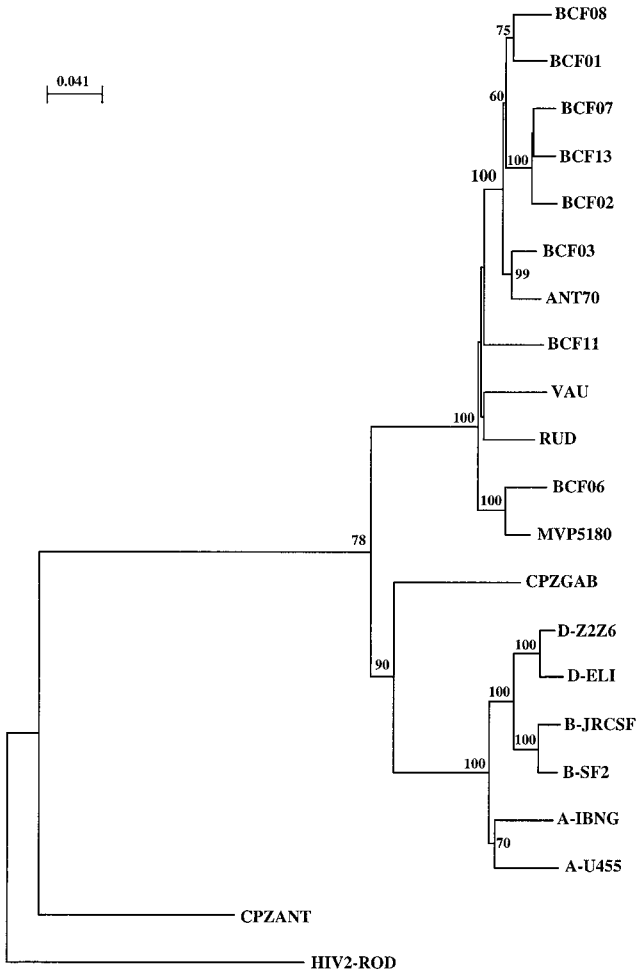

FIG. 2. Phylogenetic tree analysis comparing the amino-terminal region of the HIV-1 *pol* gene as described in Fig. 1. Tree topology was inferred by the neighbor-joining method. The tree was based on an alignment of encoding nucleotide sequences from which columns containing gaps have been deleted (1,182 nucleotides). The tree was rooted with the HIV-2 ROD sequence. The numbers given at the branch points are the 50% threshold majority consensus values for 100 bootstrap replicates. Vertical distances are for clarity only.

well as SIV CPZ sequences, were included, and the tree was rooted with the HIV-2 ROD sequence.

In the phylogenetic tree constructed with the neighbor-joining method, the branching pattern was very similar to that obtained with the *gag* sequences, namely, the cluster including BCF01, BCF02, BCF03, BCF07, BCF08, and ANT70 (13). Moreover, the tree constructed by the maximum-likelihood method gave a similar cluster ($P < 0.01$). Interestingly, this cluster corresponded to the NNRTI-resistant isolates (except isolate ANT70, which was not phenotypically tested), showing that the phenotypic susceptibility data had a molecular basis. All of the isolates forming this cluster (including ANT70) have a cysteine at position 181 of the RT gene instead of the tyrosine in the other sequenced isolates. We used the VESPA program (12) to search for a signature to discriminate between isolates resistant to all three NNRTIs and isolates resistant to only one or two of them. Lysine 28 and isoleucine 142 were conserved in isolates resistant to all three NNRTIs. In the isolates resistant to only one or two of the NNRTIs tested, in addition to tyrosine 181, valine 21, glutamic acid 247, and valine 261 in the RT gene were conserved.

The genetic distance in the *pol* gene within the group O isolates ranged between 3.3 and 12.2% for nucleotide sequences and between 3 and 14.7% for amino acid sequences, similar to the distance within group M isolates (2.9 to 11.6% and 4.3 to 14.6%, respectively). The genetic distance from group O to group M ranged from 32.7 to 37.4% for nucleotide sequences and from 30.0 to 38.3% for amino acid sequences.

The extensive HIV-1 group O variability (8, 13, 27) might raise therapeutic problems, as amino acid variability may affect some of the sites involved in conferring resistance to antiretroviral drugs. This is particularly important, as the current viral load assays used to monitor treatment efficacy fail to detect HIV-1 group O RNA sequences.

None of the HIV-1 group O isolates tested harbored substitutions at codons associated with resistance to ZDV, ddI, or ddC in HIV-1 group M isolates. The two phenotypically 3TC-resistant strains harbored the amino acid substitutions previously described in resistant group M isolates. Isolate BCF13, exposed to 3TC for 6 months, had a valine at codon 184 of the RT gene and was phenotypically highly resistant to this drug. The MVP5180 isolate unexposed to 3TC had an isoleucine at position 184. However, the clinical significance of this substitution might be affected by the fact that we used a laboratory strain cultured on the MT2 cell line and not a primary isolate.

Seven of the 11 isolates tested were naturally resistant to all of the NNRTI agents used. In six of seven cases, the sequence analyses revealed the Y181C substitution, which has been linked to resistance to NNRTI in group M isolates (22). The analysis of the *pol* sequence of the MVP5180 isolate (8), which was phenotypically resistant to NNRTI, but with lower IC_{50}s of NVP and DLV, revealed no substitution at codon 181 of the RT gene. However, this strain, as well as the BCF01, BCF06, BCF11, and BCF13 isolates, harbored a K103R substitution associated with resistance to trovirdine (16), while the K103N substitution is associated with resistance to NNRTIs in group M strains (21). In all of these cases, this substitution was associated with resistance to TIBO, while susceptibility to NVP and DLV was variable (Table 3). All of the strains exhibited the A98G substitution associated with NVP resistance in group M strains (22), but no correlation was found between this substitution and resistance to NVP in group O stains, three isolates (VAU, RUD, and BCF06) being susceptible to NVP despite this substitution.

Wild-type HIV-2 RT shows 60% sequence identity to HIV-1 RT but is not inhibited by any of the known NNRTIs (25). There is also 60% identity between HIV-1 and HIV-2 residues that point toward the NNRTI binding pocket; nonidentical residues presumably account for the diminished NNRTI activity. One of the key differences is the presence of an isoleucine instead of a tyrosine at position 181. We thus looked for similarities with HIV-2 amino acid sequences in the NNRTI binding pocket (101 to 106 and 176 to 190), but HIV-1 group O strains were closer to HIV-1 group M than to HIV-2 in critical codons. Isoleucines 181 and 179, leucine 188, and alanine 190, associated with natural resistance to NNRTI in HIV-2 (25), were never encountered in the HIV-1 group O strains sequenced.

All of the group O isolates were susceptible to SQV, and no substitutions at codon 48 or 90 of the protease gene (10) were observed. Although we found point mutations associated with resistance to RTV, no correlation was found between these genotypic modifications and phenotypic susceptibility. The sequence analyses revealed different mutation patterns characteristic of different strains. Several studies suggested strain-specific rather than group-specific patterns of protease inhibitors-induced mutations and the emergence of cross-resistant

variants during selection with protease inhibitors (17), the degree and spectrum of cross-resistance varying widely among isolates. As the selection of multiple genetic mutations, most of which are cross-resistant, seems to be a general property of this class of compound, simple genetic determination appears to be insufficient for determining the clinical significance of these mutations. Thus, phenotypic susceptibility testing appears to be the strategy of choice for identifying the emergence of protease inhibitor-resistant strains in HIV-1 group O-infected patients.

In conclusion, this study of HIV-1 group O susceptibility to antiretroviral drugs revealed that, despite the extensive sequence variability of the isolates we analyzed, these viruses present peculiar phenotypes with natural resistance to NNRTI. However, at least in part, the same mutations are associated with resistance to antiretroviral drugs in HIV-1 group M and group O strains. HIV-1 group O may thus represent a useful complementary model for the study of the mechanisms of resistance to antiretroviral drugs and to improve our comprehension of the mutations involved in the drug-induced evolution of HIV.

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