HIV-1 Group O Genotypes and Phenotypes: Relationship to Fitness and Susceptibility to Antiretroviral Drugs

Denis M. Tebit,^{1,*} Hamish Patel,¹ Annette Ratcliff,¹ Elodie Alessandri,² Joseph Liu,¹ Crystal Carpenter,¹ Jean-Christophe Plantier,² and Eric J. Arts^{1,†}

Abstract

Despite only 30,000 group O HIV-1 infections, a similar genetic diversity is observed among the O subgroups H (head) and T (tail) (previously described as subtypes A, B) as in the 9 group M subtypes (A–K). Group O isolates bearing a cysteine at reverse transcriptase (RT) position 181, predominantly the H strains are intrinsically resistant to non-nucleoside reverse transcriptase inhibitors (NNRTIs). However, their susceptibility to newer antiretroviral drugs such as etravirine, maraviroc, raltegravir (RAL), and elvitegravir (EVG) remains relatively unknown. We tested a large collection of HIV-1 group O strains for their susceptibility to four classes of antiretroviral drugs namely nucleoside RT, non-nucleoside RT, integrase, and entry inhibitors knowing in advance the intrinsic resistance to NNRTIs. Drug target regions were sequenced to determine various polymorphisms and were phylogenetically analyzed. Replication kinetics and fitness assays were performed in U87-CD4⁺CCR5 and CXCR4 cells and peripheral blood mononuclear cells. With all antiretroviral drugs, group O HIV-1 showed higher variability in IC₅₀ values than group M HIV-1. The mean IC₅₀ values for entry and nucleoside reverse transcriptase inhibitor (NRTI) were similar for group O and M HIV-1 isolates. Despite similar susceptibility to maraviroc, the various phenotypic algorithms failed to predict CXCR4 usage based on the V3 Env sequences of group O HIV-1 isolates. Decreased sensitivity of group O HIV-1 to integrase or NNRTIs had no relation to replicative fitness. Group O HIV-1 isolates were 10-fold less sensitive to EVG inhibition than group M HIV-1. These findings suggest that in regions where HIV-1 group O is endemic, first line treatment regimens combining two NRTIs with RAL may provide more sustained virologic responses than the standard regimens involving an NNRTI or protease inhibitors.

Introduction

HIV-1 GROUP M (major) dominates the global HIV epidemic making up more than 97% of all HIV infections with HIV-2 responsible for another 1%–2%.¹ Other groups such as O (outlier), N (non-M, non-O), and P were described at least a decade after group M with an epicenter in Cameroon/Gabon where group O prevalence reached 2% early in the epidemic (1990–1997).^{1–4} As the HIV epidemic progresses, group O prevalence has continued to decrease in the population with rates now as low as 0.55% in 2004 and 1% in 2008.^{2,5–8} Nonetheless, with HIV-1 prevalence at ~5% in

Cameroon, HIV-1 group O may be responsible for more than 30,000 infections.⁹

Apart from their high genetic variation, group O HIV-1 isolates show some phenotypic differences relative to HIV-1 group M. Specifically, more than 60% of group O strains are naturally resistant to non-nucleoside reverse transcriptase inhibitors (NNRTIs) such as nevirapine (NVP), efavirenz (EFV), and etravirine (ETV).^{10–12} This NNRTI resistance is caused by the presence of a cysteine at position 181 in the NNRTI binding pocket of reverse transcriptase (RT) and is analogous to the Y181C mutation selected with NVP treatment in HIV-1 group M infections.¹¹

¹Division of Infectious Diseases, Case Western Reserve University, Cleveland, Ohio.

²Laboratory of Virology, Rouen University Hospital, Rouen, France.

*Current affiliation: Myles Thaler Center for AIDS and Human Retrovirus Research, University of Virginia, Charlottesville, Virginia.

†Current affiliation: Department of Microbiology and Immunology, Schulich School of Medicine and Dentistry, Western University, London, Ontario, Canada.

In Cameroon and Gabon, high frequency of group O in the HIV-infected populations creates challenges for treatment strategies, which in best practice requires phenotypic and genotypic testing before treatment of a group O infection.^{8,13} Interestingly, EFV+emtricitabine (or lamivudine/3TC)+tenofovir (or zidovudine) are the first line regimens most commonly used across the African continent, despite pre-existing EFV resistance in ~30,000 of 600,000 HIV-1-infected patients in Cameroon.^{3,9,14} Due to the high costs in genotyping and drug resistance testing, about 1%–2% of patients in some areas of Cameroon, Gabon, and Equatorial Guinea where group O dominates will immediately fail an NNRTI-based treatment due to a HIV-1 group O infection.

Maraviroc (MVC), a CCR5 antagonist, is a relatively new drug that shows activity against group O, but has not been used routinely in sub-Saharan Africa. Earlier studies have reported that MVC in combination with two nucleoside inhibitors is similar or even better at reducing viral loads than most protease inhibitors (PIs) as well as some NNRTIs-based regimens. However, these controlled clinical studies on MVC were largely focused on HIV-1 group M subtype B-infected cohorts in high-income countries.¹⁵ Furthermore, for any MVC containing regimen to be effective, CXCR4-using HIV-1 variants must be absent in the inpatient virus population. Because group O and M share <40% sequence similarity in the V3 loop, various algorithms might not predict coreceptor usage of HIV-1 group O.^{16–18}

Previous studies indicate that most HIV-1 group O isolates may show limited susceptibility to protease inhibitors due to the presence of secondary PI resistance mutations (10I, 15V, 36I, 41K, 62V, 64V, 71V, and 93L) in most strains and might also be difficult to manage.^{13,19} In fact, two case studies reported rapid resistance upon treatment of group O-infected individuals with PI-based regimens.²⁰ The integrase strand transfer inhibitors (INSTIs) namely elvitegravir (EVG), raltegravir (RAL), and dolutegravir (DTG) may therefore hold a promising future in Africa as they can inhibit both HIV-1 groups (M and O) and HIV-2.^{21–25}

Recently, Leoz *et al.* suggested a novel classification of group O based on a clustering pattern into H (head) and T (tail). These two clusters were further subdivided into H1, H2, H3, and T1 and T2, respectively.¹² Our previous study suggested that an NNRTI-resistant and -sensitive phenotype in group O could be distinguished into two subgroups based on the presence of a cysteine or tyrosine at amino acid position 181 (C181 and Y181). Notably, there is a strong association between subgroup H and the cysteine at position 181 of RT. The majority of H strains (80.5%) comprise either H1 or H2 and are grouped as C181-like.^{11,12}

To understand the importance of the C181 and Y181 clusters, we tested a large cohort of 18 C181 and Y181 HIV-1 group O isolates, 1 O/M recombinant isolate, and 8 group M isolates (as controls) for susceptibility to various RT inhibitors (3TC, NVP, ETV), integrase (IN) inhibitors (RAL and EVG), and entry inhibitor (MVC). Susceptibility to these inhibitors were compared to sequences from Pol and Env of these group O strains to identify any known drug resistance mutations, previously identified in group M subtype B HIV-1. Coreceptor usage determination for group O HIV-1 isolates was performed with *in silico* group M web-based phenotypic algorithms to predict coreceptor usage. Finally, dual virus competition assays in peripheral blood mononuclear cells (PBMCs) were used to determine replicative fitness of the

group O HIV-1 isolates and investigate a possible correlation with drug susceptibility.

Materials and Methods

Cells and viruses

U87 human glioma cells expressing CD4 and either CXCR4 or CCR5 were obtained from the AIDS Research and Reference Reagent Program. PBMCs were obtained from whole blood of healthy HIV-negative donors using the Ficoll-hypaque centrifugation method. PBMC cultures were stimulated with 1 µg/ml phytohemagglutinin (PHA; Gibco) for 48 h in RPMI 1640 medium (Gibco) containing 10% fetal bovine serum and 100 µg/µl penicillin–streptomycin. All viruses used in this study were derived from primary isolates with the exception of CMO2.41, which was grown from an infectious molecular clone (Table 1).²⁶ These viruses, including 9 group M (2 each of subtypes A, C, D and 3 subtype B) and 18 group O and 1 O/M recombinant, were titrated on PBMCs and titers determined by the Reed–Muench end point titration method.²⁷ One of the subtype B virus (B10) was used only for fitness and not for drug susceptibility studies. Eight of these group O isolates were provided by the AIDS Reagent Program.

Antiretroviral drugs and drug sensitivity assays

The antiretroviral drugs 3TC, NVP, ETV, MVC, and RAL used in this study were obtained from the AIDS Research and Reference Reagent Program. EVG was obtained from a local pharmacy, but its fifty percent inhibitory concentrations (IC₅₀) were checked and found to be similar to those of purified EVG. Briefly, cells were added onto a 96-well plate at a concentration of 1×10^4 cells per well and allowed to adhere overnight. On the next day, cells were treated with various drugs for 1 h before addition of virus at a multiplicity of infection (MOI) of 0.01 infectious units per milliliter. The virus was washed off 24 h postinfection with phosphate-buffered saline, and a fresh medium containing drug was added. Aliquots of the supernatants were then collected at intervals from days 3 to 10 and virus production quantified by radioactive RT assay. IC₅₀ curves were constructed using nonlinear regression curve fitting features of GraphPad Prism 5 software.

Coreceptor determination and replication kinetics

The biological phenotype [syncytium inducing (SI) or CXCR4 tropism and nonsyncytium inducing (NSI) or CCR5 tropism] for each virus was determined by infecting U87.CD4 cell lines expressing either CXCR4 or CCR5 receptors. Each HIV-1 isolate in the present study was used to infect 25,000 U87/CD4/CCR5 and U87/CD4/CXCR4 cells in duplicate in a 48-well plate as previously described.²⁸ Virus production was determined by assaying the RT activity in the cell-free supernatants at different time points after infection, as well as monitoring for syncytium formation in each of the cell lines.

Ex vivo growth competition assays

Ex vivo pathogenic fitness assays were performed as previously described.^{11,29–32} Full pairwise dual infection/competition was performed with 20 HIV-1 primary isolates (18 group O; 1 O/M recombinant), Table 1, and 1 subtype B isolate that was used previously in a larger set of group O versus M competitions.³⁰ Competitions were performed in 2×10^5

TABLE 1. CHARACTERISTICS OF HIV-1 GROUP O PRIMARY ISOLATES USED FOR DRUG SUSCEPTIBILITY AND FITNESS ASSAYS

<i>Virus</i>	<i>Group O cluster^a</i>	<i>Titer (PBMCs)</i>	<i>Coreceptor usage/phenotype</i>	<i>NNRTI mutations</i>
YBF39	H3	10 ^{3.5}	CCR5/NSI	G98, E179
BCF03	H1	10 ^{3.75}	CCR5/NSI	G98, E179, C181
YBF26	H	10 ^{3.5}	CCR5/NSI	G98, E179, C181
BCF01	H	10 ³	CCR5/NSI	G98, R103, E179, C181
BCF07	H	10 ^{4.25}	CXCR4/CCR5/Dual	G98, E179, C181
BCF13	H1	10 ^{2.5}	CCR5/NSI	G98, C181
RBF189	H3	10 ^{3.5}	CCR5/NSI	G98, E179, C181
YBF38	H	10 ^{4.5}	CCR5/NSI	G98, E179, C181
YBF32	H	10 ^{3.5}	CCR5/NSI	G98, E179, C181 (T215IT-NRTI)
BCF02	H1	10 ^{3.5}	CCR5/NSI	G98, E179, C181
CMO2.41	H	10 ^{4.5}	CCR5/NSI	G98, E179, C181
YBF125	T1	10 ^{3.75}	CCR5/NSI	G98, R103, E179
BCF11	T1	10 ^{2.75}	CCR5/NSI	G98, E179
YBF16	T2	10 ^{4.5}	CCR5/NSI	G98, E179
YBF37	T	10 ^{3.5}	CCR5/NSI	G98, E179
YBF35	T1	10 ^{2.5}	CCR5/NSI	G98, E179
BCF06	T1	10 ^{4.5}	CXCR4/CCR5/Dual	G98, E179
MVP5180	T1	10 ^{3.5}	CXCR4/CCR5/Dual	G98, E179
RBF208 ^b	H	10 ^{3.5}	CCR5/NSI	G98, E179, C181

^aClassification was based on the recent proposal by Loez *et al.*¹²

^bRBF208 is an O/M recombinant; its RT is group O (H cluster), while its envelope is group M (subtype D, see Table 2).

NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; NSI, nonsyncytium inducing; PBMC, peripheral blood mononuclear cell; RT, reverse transcriptase.

PHA-stimulated PBMCs by adding both viruses at an equal MOI of 5×10^{-4} , a standard well established in over 20 articles on HIV fitness.^{11,29–32} A monoinfection representing each of the viruses in the competition was included at the same MOI. Virus production was monitored by an RT assay using cell-free supernatants. At peak RT activity (10 days post-infection), cells and supernatants were harvested and stored at -80°C . DNA was extracted from harvested cells using a Qiagen DNA extraction kit.

Polymerase chain reaction and estimation of viral fitness

Envelope (Env) nested polymerase chain reaction (PCR) was performed for group O competitions and Pol PCRs for group O versus M competitions. The group O Env first round primers used were Env-O-N-F2 and Env-O-N-R2, while the second round primers were Gp O-N-M-P E80 and Gp O-N-M-P E125. The pol primers used to amplify group M and O DNA had been described previously.²⁹ Briefly, M/O RTS1 to M/O RTA4 and M/O RTS2 to M/O RTA3 as first and second round forward and reverse primers, respectively. Virus production in dual infection/competition assays was quantified using a heteroduplex tracking assay (HTA) as previously described.^{11,29–33} Briefly, radiolabeled DNA probes (two group O strains and one group M) were also amplified using the same set of primers in the *env* and/or *pol* RT regions. The antisense primer for these amplifications was end labeled with 5' [³²P]ATP as described elsewhere.³¹ Radiolabeled PCR-amplified DNA probes were separated on 1% ethidium bromide-stained agarose gels and purified with a QIAquick Gel Extraction Kit. Purified probe (300 cpm) was mixed with 5 μl of PCR products in the presence of annealing buffer and loading dye. This mixture was denatured at 95°C for 3 min, annealed at 37°C for 5 min, and then kept on ice during loading on a 6% nondenaturing polyacrylamide gel. Het-

eroduplexes corresponding to each HIV-1 isolate in a dual infection and mono infections were identified by using a Molecular Imager FX (Bio-Rad) phosphor imager and then quantified by using Quantity One software. The relative viral fitness and fitness difference were estimated by HTA analysis as described previously.^{29–32} The final ratio of the two viruses produced from each dual infection was determined by comparing the virus production in the competition to the virus production in the monoinfection. Production of each HIV-1 isolate in a dual infection (f_0) was divided by the initial proportion in the inoculum (i_0) to determine the relative fitness ($W = f_0/i_0$). The fitness difference (W_d) is the ratio of the relative fitness values of each HIV-1 isolate in the competition ($W_d = w_M/w_L$).^{11,29–33}

Sequence analyses

Proviral DNA was extracted from the cells from the replication kinetics experiments, and the RT [position 2550–3239 in HxB2 (690 nucleotides)], IN [position 4230–5096 in HxB2 (867 nucleotides)], and Env gp120 [position 6213–7805 in HxB2 (1,623 nucleotides)] of the group O viruses were PCR amplified in a nested PCR and purified by the QIAquick PCR purification kit (Qiagen) and sequenced with forward and reverse primers from the nested PCR. Sequence chromatograms were manually edited for alignment using Vector NTI. Sequence and neighbor joining phylogenetic trees were constructed using Clustal X and viewed with Tree View.³⁴

Prediction of HIV-1 coreceptor usage and glycosylation sites by web-based algorithms

Web-based bioinformatics coreceptor prediction programs were tested for their ability to determine the coreceptor usage of HIV primary isolates based on V3 loop sequences (HXB2 gp160 amino acids 296–334). These programs that are based

on group M subtype B V3 loop sequences included the position-specific scoring matrix (PSSM) and geno2pheno [coreceptor] (g2p).^{35,36} PSSM analysis was performed using subtype B X4R5 matrix, whereas for g2p, significance levels were set to the optimized cutoffs based on clinical analyses (2% and 5.75% false-positive rates).³⁷ Prediction was also performed by manually detecting the presence of positively charged amino acids at codons 11 and/or 25 (11/25 rule) of the V3-loop (HXB2 gp160 positions 306 and 322, respectively). Potential N-linked glycosylation sites were predicted by manually identifying the NxT and NxS sequences in the V3 loop and confirmed by using an online tool that provided the sum of potential N-linked glycosylation in the envelop gp120.³⁸

Statistical analyses

Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software). Two tailed Student's *t*-tests were

used to compare the mean IC₅₀ between groups M and O. All tests were considered significant if *p* < .05.

Results

HIV-1 group O isolates and phylogenetic classification

The 18 group O primary isolates used for this study have been partially characterized in previous studies.^{10,39,40} Prior genotypic classification of these isolates typically involved the use of envelope sequences due to its high genetic diversity.^{10,39,40} However, other regions such as the RT have been shown to be effective in group O genotypic classification.^{11,41} The phylogeny of the group O strains were determined for the first ~700 base pairs of RT (Fig. 1A), the entire integrase (Fig. 1B), and the envelope gp120 sequences of the 18 primary isolates (Table 1). Using a limited set of 47 sequences, we previously suggested the separation of HIV-1 group O into 2 clusters based on the C181 and Y181 residues in the

FIG. 1. Phylogenetic trees of HIV-1 group O primary isolates (A) reverse transcriptase (B) integrase. Sequences were aligned with reference HIV-1 group O, N, P, and SIVgab sequences from the HIV Los Alamos Database using the neighbor joining method and mapped onto a tree using Tree View (1,000 bootstraps). The bootstrap values >700 were considered significant and are shown for the various HIV-1 groups and the group O sub clusters. Group O isolates were distinguished either as C181-like or Y181-like. Their respective subgroups (H, H1, or H3) and (T, T1, and T2) are shown in brackets after each isolate name.

NNRTI binding pocket of RT corresponding to NNRTI-resistant and -sensitive phenotypes, respectively. However a recent study comparing 190 group O sequences suggested a more detailed classification into subgroups H (H1, H2, and H3) and T (T1 and T2). Although not applicable in all cases, C181 in RT shows a strong association with the H subgroup among group O strains.¹² Eleven of the group O isolates in our study were C181-like and clustered with the H strains, including the prototype group O isolate Ant70, while 7 were Y181-like and grouped with T strains and the reference MVP5180 strain (Fig. 1A, B and Table 1). All the group O isolates formed concordant clusters in neighbor joining trees constructed with the RT, integrase, and Env gp120 coding sequences. Interestingly, the C181 and Y181 residues remained the most conserved in any HIV-1 coding region within these clusters. Exceptionally, the O/M recombinant RBF208 strain clustered with H1 subgroup in pol (Fig. 1A, B and Table 1), but with subtype D-group M in the envelope (Table 2).

Susceptibility of HIV-1 group O strains to RT inhibitors

Multiple cycle drug susceptibility assays were performed in U87.CD4.CCR5 or CXCR4 cells in the presence of 10-fold decreasing concentrations of drugs starting at 10 or 100 μ M depending on the drug type (Fig. 2). Eighteen group O and 8 group M strains, at least 2 from each of the subtypes A, B, C, and D, were tested for susceptibility to 3TC [nucleoside reverse transcriptase inhibitor (NRTI)], NVP, ETV (NNRTI) RAL, EVG (INSTI), and MVC (entry inhibitor). As expected, all group O and M strains were inhibited by 3TC with a mean IC_{50} value of 48.7 nM for group O (range 2.2–90 nM) and of 59.3 nM for group M (range 18–137 nM). There was no statistical difference in the 3TC IC_{50} values per group or subtype. On the contrary, inhibition of group O strains by NVP and ETV was variable (NVP mean IC_{50} = 6,174 nM, range 19.2–10,000 nM; ETV mean IC_{50} = 39.8 nM, range 0.4–311 nM) depending on the presence of tyrosine or cysteine at position 181 of RT as well as lysine or arginine at position 103 (Fig. 2). There was a significant difference ($p < .005$) in NVP susceptibility between the group M and O isolates as a result of this presence of C181 and/or R103 (Fig. 2C-i, ii). We previously showed that the R103 genotype causes a >10-fold resistance to NNRTIs and is enhanced by the presence of G98 and E179, both naturally occurring polymorphisms in group O.¹¹

As described above and in previous studies, at least 60% of all group O HIV-1 in the infected populations are intrinsically resistant to NNRTI. The level of intrinsic NNRTI resistance in group O virus is relatively comparable to that observed in HIV-1 group M isolates acquiring NNRTI resistance during treatment.

Susceptibility of HIV-1 group O to MVC inhibition

MVC is seldom prescribed as a first line treatment regimen for HIV-1-infected patients in the developed world, despite potent antiviral activity and low toxicity. It is used almost as a last resort for salvage therapies due to increased frequency of the CXCR4-using HIV-1 that emerges in late disease and is intrinsically resistant to MVC. We have shown a significant variation of nonsubtype B HIV-1 isolates to inhibition by MVC with IC_{50} values varying >100-fold.^{42,43} In this study, all 8 group M, 1 O/M, and 18 group O strains were

susceptible to MVC inhibition. However, higher variations in MVC IC_{50} values were observed with the group O HIV-1 isolates (mean IC_{50} = 51.2 nM, range 1–315 nM) than with the group M isolates (mean IC_{50} = 32.4 nM, range 2–102 nM) (Fig. 2B). All group O isolates tested for MVC sensitivity were CCR5-tropic based on infections of CD4⁺ U87 cells expressing either CXCR4 or CCR5 (Tables 1 and 2).

Higher variability of group O isolates to MVC inhibition may be related to specific features in the gp120, especially the V3 loop. To determine if any polymorphism in envelope could map to reduce susceptibility, we sequenced the entire envelope gp120 of these isolates and compared these sequences to any mutations or polymorphisms related to variable inhibition by MVC or other CCR5 antagonists/agonists.^{42–47} Specifically six amino acid positions namely 33 and 117, 290, 315, 396, 425 that span the C1, C2, V3, V4, and C4 regions of gp120, respectively, were analyzed (Table 3). Compared to group M, the amino acid residues at these positions showed a high variation in the group O sequences with less than 5% similarity with the exception of Q290, which was 100% conserved in group O. At position 117,⁴² arginine was replaced by a lysine in all the group Os while at amino acid position 425⁴² and asparagine was replaced by an arginine in almost 90% of group O (Table 3). A N425K mutation conferred a high level MVC resistance in a group M-A isolate.⁴²

Predicting coreceptor usage among group O HIV-1 isolates

CCR5 tropic HIV-1 strains are typically transmitted from donor to recipient, establish a new infection, and predominate during asymptomatic disease. MVC is an effective inhibitor of CCR5-using HIV-1 isolates, whereas CXCR4-using HIV-1 appears late in disease and is MVC resistant. Infection by CCR5-using HIV-1 and the late appearance of CXCR4-using HIV in disease have also been studied in patients infected with HIV-1 group M. However, the prevalence and evolution of HIV-1 using specific coreceptors in group O infections are still poorly understood as only very few group O-infected subjects have been followed during disease.⁴⁸ This of course also has relevance to the use of MVC in group O-infected individuals. To further our knowledge on coreceptor usage among group O isolates, U87.CD4.CCR5 or CXCR4 cells were exposed to each of the 18 HIV-1 group O primary isolates and 1 group M/O (Tables 1 and 2). Of the 18 group O isolates, 15 were CCR5 tropic, while 3 (BCF07, BCF06, and MVP5180) were dual/mixed tropic with a higher level of infection in the U87.CD4.CXCR4 (Fig. 3A and Tables 1 and 2).

To correlate genotype to phenotype (tropism), V3 loop sequences of these group Os and the O/M primary isolates were analyzed by various algorithms predicting coreceptor usage (Geno2Pheno, PSSM, net charge rule, and 11/25 rule) and then compared to actual tropism on the U87.CD4.CCR5 or CXCR4 cells. To increase the statistical power, we also included sequences from nine group O isolates (Table 2) that had previously been characterized for coreceptor usage (some kindly provided by Dr. Lutz Guertler, pers. Comm.).⁴⁹ We suspected that at least the PSSM and Geno2Pheno would fail in coreceptor prediction due to higher nucleotide genetic diversity in the V3 loop of HIV-1 group O. Earlier studies had shown the poor predictions of PSSM for nonsubtype B isolates in the M group.⁵⁰ The group O V3 loops are also longer, ranging

FIG. 2. Susceptibility of HIV-1 group O and M isolates to different classes of inhibitors. (A) NRTI (Lamivudine-3TC); (B) entry inhibitor (MVC); (C) NNRTI, (i) NVP and (ii) ETV; (D) INSTI (i) RAL and (ii) EVG. NVP an NNRTI and EVG an INSTI show significant differences in their potency against group O versus M. The NNRTI difference is due mostly to the natural resistance mutation, Y181C, while the reason for the EVG resistance is unknown. ETV resistance is also observed among the C181 isolates. Although not significant, a few group O strains are less susceptible to MVC. The mean IC_{50} as well as the average around the mean are indicated in the figures. ETV, etravirine; EVG, elvitegravir; INSTI, integrase strand transfer inhibitor; MVC, maraviroc; NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; NVP, nevirapine; RAL, raltegravir.

from 34 to 39 amino acids compared to the 34–35 in group M (Table 2). Based on actual biological phenotyping on U87-CD4⁺ cells expressing either CCR5 or CXCR4 receptors, three group O strains BCF06, MVP5180, and BCF07 were dual tropic with an overwhelming dominance of the X4 phenotype

(Fig. 3A and Table 2) while MVP2171 had been previously shown to be X4 tropic.⁴⁹ Both PSSM and Geno2Pheno predicted the majority of group O V3 sequences as X4, despite only four X4-using group O viruses (using V3 loop sequences trimmed to 35 aa or untrimmed). Overall, all four algorithms

TABLE 3. COMPARISON OF HIV-1 GROUP O GP120 SEQUENCES (POSITIONS 33, 117, 290, 315, 396, AND 425) FROM THE LOS ALAMOS HIV DATABASE AND THOSE ANALYZED IN THIS STUDY

Mutation (group M)	Residue group O	Los Alamos group O (n=27)	Sequences from this study (n=17)
		Amino acid frequency (%)	Amino acid frequency (%)
E33G	R	3 (11.1)	1 (5.9)
	K	9 (33.3)	8 (47)
	S	6 (22.2)	3 (17.3)
	N	5 (18.5)	1 (5.9)
	D	3 (11.1)	2 (11.8)
R117Q	E	1 (3.7)	2 (11.8)
	K	27 (100)	17 (100)
Q290K	Q	27 (100)	17 (100)
Q315R	A	20 (74)	8 (47)
	W	3 (11.1)	0 (0)
L396V	S	2 (7.4)	7 (47)
	R	1 (3.7)	2 (7.4)
	S	10 (37)	5 (29.4)
	I	4 (14.8)	2 (11.8)
	K	6 (22.2)	3 (17.3)
	T	3 (11.1)	2 (11.8)
	D	1 (3.7)	0 (0)
	F	2 (7.4)	0 (0)
	N	1 (3.7)	3 (17.3)
	M	0 (0)	1 (5.9)
	L	0 (0)	1 (5.9)
	R	27 (100)	15 (88.2)
N425K	K	0 (0)	1 (5.9)
	N	0 (0)	1 (5.9)

(Geno2Pheno, PSSM, net charge rule, and 11/25 rule) largely failed to predict CXCR4 or CCR5 usage. The 11/25 rule had the highest congruence with coreceptor usage, but only 16 of 23 CCR5-using group O isolates were predicted to be CCR5 tropic, whereas only 1 of 4 CXCR4-using group O isolates was predicted as X4 tropic (Table 2). Finally, the WetCat program, another web-based algorithm, could not predict the tropism of any group O isolate as it did not consider any of the input sequences as V3 loop sequences.

When considering that the 11/25 rule predicted coreceptor usage (16/27) only slightly higher than chance, it is possible that other V3 loop sequences may be more prognostic of the X4 phenotype. Nine of the 23 V3 loop sequences of CCR5-using group O viruses had a positively charged amino acid (Arg or Lys) at position 11 or 25 (and never linked); Table 2. In contrast, less than 5% of all CCR5-using HIV-1 group M isolates have a positively charged amino acid at position 11 or 25.^{33,51,52} Unlike conserved GPGX found at the tip of the group M V3 loop, group O HIV-1 has a less conserved GPMA at the tip (Table 2). Interestingly, three of the four X4 HIV-1 group O isolates (BCF06, MVP5180, and MVP2171) contained an Arg either as the fourth residue or immediately following the GPM(A/S) tip sequence. The presence of Arg was suggestive of and therefore considered the most predictive amino acid of X4 versus R5 usage (Table 2).

Susceptibility of HIV-1 group O to inhibition by integrase inhibitors

Due to the potential treatment failure of NNRTIs, MVC, and PIs in group O infections, we tested the sensitivity of our group O HIV-1 panel to integrase inhibitors, RAL and EVG. In contrast to the high variability in IC₅₀ values to MVC and the intrinsic NNRTI resistance, group O HIV-1 showed similar sensitivity to RAL inhibition as did the group M HIV-1 isolates (compare IC₅₀ values = 40.1 ± 7.6 to 23.7 ± 3.8 nM, respectively); Figure 2D-i as shown previously with a limited number of group O isolates.⁵³ In addition, the range in these group O IC₅₀ values to RAL was less than 10-fold, that is, similar to that observed with 3TC. However, the mean IC₅₀ values to EVG were significantly higher among the group O than group M isolates (IC₅₀ values of 0.14 nM vs. 1.57 nM, respectively, *p* = .008 two tailed *t*-test). Unlike the narrow range of RAL IC₅₀ values, the IC₅₀ values for EVG ranged from 0.03 to 7.2 nM or >100-fold with the same group O isolates (Fig. 2D-ii). We have obtained IN sequences for all the group O isolates and have yet to identify specific polymorphisms that may explain for these extreme differences in EVG sensitivity.

HIV-1 group O isolates have similar replicative kinetics and fitness that do not correlate with drug susceptibility

As a follow-up of our previous study involving the C181 and Y181 subclusters in group O, we continued to measure replication kinetics in monoinfections of U87.CD4.CCR5 (or U87.CD4.CXCR4) and replicative fitness in dual virus competitions with the additional 18 group O isolates in this study. As shown in Figure 3A, there was no significant difference between the C181 and Y181 clusters on either U87.CD4.CCR5 or CXCR4 cells, but a slight increase (not significant) in C181 replication when grown on PBMCs (Fig. 3B). The SI group O viruses replicated faster in the U87.CD4.CXCR4 cells than did the NSI group O viruses in the U87.CD4.CCR5 cells (Fig. 3C). Similar results have been repeatedly reported with SI versus NSI group M HIV-1 isolates.^{30, 31,33}

Replicative fitness is best measured in *ex vivo* dual virus competitions performed in primary cells such as PBMCs. To this end, R5 (or X4) group O isolates were competed against each other and with the reference group M subtype B (B10) isolates in PBMC. The group M subtype B isolate (B10) had been previously shown to have very low replicative fitness compared to other group M isolates,³⁰ and yet, the group M-B10 HIV-1 outcompeted 12 of 15 group O R5 HIV-1 isolates and supporting previous studies showing lower group O versus M HIV-1 fitness.²⁹ The total relative fitness of the group O isolates was grouped according to C181 or Y181 subgroup (Fig. 4A), but as shown, the mean relative fitness value between the group O subgroups was not significantly different. In these studies, the maximal replication fitness is 2, which indicates that one virus completely outcompeted the other (see Materials and Methods section).^{11,29–31} A mean relative fitness of 1 indicates that this particular virus was on average as fit as all the other group O isolates. Three R5-using isolates (RBF189, BCF03, and YBF39) had high mean fitness values (1.02, 1.11, and 1.01, respectively) (Fig. 4A). In general, the mean relative fitness value correlated with the replication kinetics in monoinfections (*r* = 0.65, *p* < .052, PBMC). However, we have previously shown that variation

FIG. 3. Replication kinetics and biological phenotype (tropism) of various HIV-1 groups (**A**) All viruses were grown in both U87.CD4.CCR5 and U87.CD4.CXCR4 cell lines for 10 days. Supernatants were collected on days 3, 5, 8, and 10. The values shown above are the cumulative average with the highest represented as 2. *Black* and *gray* represent X4 and R5 tropism, respectively. (**B**) Replication kinetics comparison by C181-like (*black*) and Y181-like (*gray*); (**C**) viral tropism (phenotype), SI (*black*), and NSI (*gray*). NSI, nonsyncytium inducing; SI, syncytium inducing.

in these correlations relate more to high variance in replicative kinetics in monoinfections.

Several studies have suggested that replication kinetics/fitness may correlate to sensitivity to antiretroviral drug inhibition. We have observed a weak correlation between replicative fitness of group M HIV-1 isolates and sensitivity to CCR5 antagonist/agonists, such as MVC, but not to NNRTIs or PIs.^{43,54} For this study, the IC₅₀ values of group O HIV-1 isolates to each drug were compared to the mean replicative fitness values of these same group O isolates (in the absence of drug). We did not observe a significant correlation, even when comparing IC₅₀ values for MVC and replicative fitness of the group O isolates (data not shown).

Discussion

Group O HIV-1 is generally less studied than group M, and at times, there are assumptions that it replicates, causes similar disease, and responds to treatment like group M HIV-1. However, HIV-1 group O shares only about 50% sequence similarity with group M, and many of these genetic differences relate to altered phenotypic properties such as interaction with Cyclophilin A/Trim5- α , tetherin antagonism, and pattern of drug resistance development.^{55,56} Over the past 10 years, HIV-1 group O has often been used as the diverse outlier virus to test the impact of newly discovered restriction factors inhibiting and cellular factors necessary for HIV-1 group M replication.⁵⁶ However, little attention is focused on the treatment of group O-infected patients in Gabon and

Cameroon, the epicenter of group O. Within the last few years, studies on group O have highlighted its important role in the evolution of HIV-1. A recent report suggested that group O strains cannot be grouped into subtypes like group M, but can be classified into two subgroups H (H1, H2, and H3) and T (T1 and T2).¹² With previous knowledge that the presence or absence of a tyrosine or cysteine at position 181 of RT (Y181 and C181) relates to sensitivity to NNRTIs and the high intrinsic resistance of the C181 isolates (~60% of all circulating group O virus),¹¹ we examined the susceptibility of 18 group O strains and an O/M recombinant virus to 3TC, NVP, ETV, MVC, RAL, and EVG relative to 8 group M isolates representing subtypes A, B, C, and D. Although group O and M HIV-1 isolates showed similar average susceptibility to the CCR5 antagonist MVC, the concentration range for group O inhibition was at least one log greater than with group M isolates. Furthermore, coreceptor usage of HIV-1 group O strains could not be predicted based on the V3 loop sequence and the current algorithms. Considering MVC treatment often fails with even a low frequency of CXCR4-using virus in the inpatient HIV-1 population, the use of MVC with group O HIV-1 infections is unlikely unless patient samples are screened with a cost-prohibitive phenotypic assay. Our drug susceptibility studies with group O HIV-1 suggest that only the integrase inhibitor, RAL, may be effective as a backbone in combination treatments with two NRTIs.

Studies on a limited number of patients infected with HIV-1 group O suggest favorable virologic control with recently approved antiretroviral agents, including the NNRTI—

FIG. 4. Fitness of HIV-1 group O and M primary isolates (**A**) Mean relative fitness of all primary isolates obtained by pairwise competitions. The isolate B10 (*black bar*) was used as the group M reference isolate. *Light gray bars* represent an O/M recombinant strain. Group O C181 and Y181 are shown as *black* and *gray bars*. (**B**) Mean relative fitness of C181 and Y181 viruses shown as *black* and *gray bars*, respectively. (**C**) Comparing the mean relative fitness of group O SI (*black*) versus NSI (*gray*) strains. Only group O strains were included in (**B**, **C**).

ETV, INSTI, and entry inhibitors in combination with two NRTIs.^{21,57–59} However, some of these studies with a limited number of patients do not provide a comprehensive picture of group O responsiveness to these new drugs. For example, most of the patients treated with ETV were infected with the group O Y181 viruses, which are not resistant to NNRTIs.⁵⁷ Despite clear recognition that 60%–70% of group O infections are intrinsically resistant to NNRTI (e.g., C181), there is no prescreening for optimal treatment for these patients and an NNRTI-based regimen is still preferred over the more challenging PI-based treatment regimens. Serological discrimination of group M and O strains using enzyme-linked immunosorbent assays is commonly performed in Central Africa where group O is most prevalent. However, even when group O is typed in Cameroon and other countries in this region, a subsequent screening of group O-infected patients for NNRTI-resistant sequences (C181 vs. Y181) before initiation for first line treatment is quite costly and impractical with current infrastructure. This obstacle could be surpassed by the development of a simple and cheap assay that could differentiate HIV-1 group O C181 from Y181 strains. Furthermore, we suggest a thorough analysis of susceptibility to approved antiretroviral drugs to identify the most potent at inhibiting diverse HIV-1 group O isolates and with the least variance in this inhibition. Considering that optimal dosing of these drugs was only assessed in the group M subtype B-infected patients, we also propose the concentrations for 50% and 90% inhibition should be similar for HIV-1 group O compared to group M:B isolates.

As described above, the mean MVC IC₅₀ concentrations for R5-using HIV-1 group O and group M inhibition were similar, but the variation in MVC IC₅₀ values was >300-fold with group O and ~100-fold with group M HIV-1. This high variation in MVC inhibition could be related to the high diversity observed within the envelope of group O isolates. In the absence of coreceptor switch to X4 usage, MVC resistance is typically associated with multiple amino acid mutations in both gp120 and gp41, which result in the ability of the MVC-resistant HIV-1 to utilize the drug-bound CCR5 receptor for host cell entry.⁴³ Most drug-resistant HIV-1 isolates (e.g., Y181C HIV-1) replicate at higher concentrations of drug (e.g., NVP) than the wild-type virus due to reduced drug binding to viral target (e.g., RT). This drug resistance mechanism results in a shift in the drug susceptibility curve and increase in IC₅₀ values. With MVC resistance caused by drug pressure, there is no significant shift in the IC₅₀ concentrations, but rather the inability of MVC to block all HIV-1 replication, regardless of the drug concentration described as the maximal percent inhibition (MPI) effect. Ten of 18 group O HIV-1 isolates showed increased sensitivity to MVC inhibition compared to group M isolates with a mean IC₅₀ value of 3.9 nM. Five group O isolates had similar sensitivity to MVC inhibition as did group M HIV-1. However, two group O isolates, representing C181 and Y181, were intrinsically resistant with MVC IC₅₀ values >200 nM. Interestingly, this intrinsic resistance to MVC was not due to an MPI, but rather a shift in the drug susceptibility curve and increase in IC₅₀ values.

Due to the diverse susceptibility of group O strains to MVC (described below), inability to predict coreceptor usage with current algorithms, and natural resistance to NNRTIs, we examined if group O HIV-1 had similar susceptibility to integrase inhibitors as group M HIV-1. RAL has been recently used in combination with NRTIs to treat HIV-1 group O patients who harbored a multiclass-resistant virus.^{21,23} In our study, HIV-1 group O isolates were slightly less sensitive to RAL inhibition than HIV-1 group M isolates (IC₅₀ values of 40.1 nM vs. 23.7 nM, respectively). However, this difference was not significant. In addition, the range of RAL IC₅₀ values for the group M and O viruses was similar and minimal compared to the >300- and >500-fold range in the MVC and ETV IC₅₀ values (respectively) observed with group O HIV-1. RAL and EVG have similar mechanisms of IN inhibition, bind to overlapping IN sites, and share similar mutations conferring resistance in HIV-1 group M subtype B isolates.^{25,60} Thus, it was surprising to note a more significant 10-fold decrease in susceptibility of HIV-1 group O versus group M HIV-1 to EVG inhibition. Of greater concern was the >200-fold range in EVG IC₅₀ values with group O isolates compared to <30-fold with group M isolates. It is not clear why the HIV-1 group O isolates are more resistant and have a greater range of sensitivity to EVG versus RAL. We did not identify common drug-resistant mutations in group O sequences, which was unlikely considering the normal susceptibility to RAL. Based on the more resistant versus more sensitive group O isolates to EVG, we are now testing numerous amino acid changes in IN that might be associated with intrinsic EVG resistance in group O isolates.

Low replicative fitness of primary HIV-1 isolates can increase susceptibility to some antiretroviral drugs. For example, we have previously shown a direct but weak correlation between relative inhibition of RANTES derivatives and the replicative fitness of primary HIV-1 isolates in primary T cells and cell lines.^{28,54,61} There is an assumption in the literature that this correlation may extend to other antiretroviral drug classes. In this study, we determined the replicative fitness of the CCR5-using group O isolates and did not observe a significant difference related to group (C181 vs. Y181) or a correlation between IC₅₀ values to any drug. Interestingly, the group O isolates, on average, show a slight decrease in susceptibility to all drugs (except 3TC), and yet, group O HIV-1 isolates are generally less fit than any group M isolate.^{29,30} Based on the lack of correlation with replicative fitness, we suspect that divergent group O evolution has led to diverse HIV-1 isolates with reduced and varying susceptibility to most antiretroviral drugs that were initially screened and selected based on inhibition of HIV-1 group M, subtype B isolates. Furthermore, this divergent evolution of group O may have led to differences in fitness even in situations where similar mutations are observed in the context of HIV-1 group M. As reported previously, the NNRTI mutations (Y181C and K103N) seem to have different fitness cost depending on the viral backbone (either group O or M).¹¹

In summary, our findings suggest that group O HIV-1 isolates show a high degree of variable susceptibility to most antiretroviral drug classes, but are highly sensitive to NRTIs. At least 60% group O patients harbor C181 isolates that are intrinsically resistant to NNRTI that includes ETV. Thus, the CCR5 antagonist, MVC, or INSTI (RAL, EVG, or DTG) would

provide a better backbone for treatment in combination with two NRTIs. In this study, MVC has been ruled out due to the range of susceptibility by group O isolates to this drug as well as the inability of the current algorithms to predict coreceptor usage. Although EVG was also ruled out due to this high susceptibility range, RAL inhibited all group O isolates with a similar potency as observed with group M HIV-1. These findings suggest that RAL-containing combinations should also be included as first line treatment regimens for group O infections in Cameroon, Gabon, Nigeria, and Equatorial Guinea. This may result in better treatment outcomes than an NNRTI-containing treatment regimen when considering that >30,000 people in Central and West Africa are infected with HIV-1 group O and are intrinsically resistant to NNRTIs. When this study was initiated, DTG was not available for testing, but we are now screening inhibition of group O HIV-1 isolates to this drug.

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Author Disclosure Statement

No competing financial interests exist.

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Address correspondence to:

Denis M. Tebit
 Myles Thaler Center for AIDS
 and Human Retrovirus Research
 University of Virginia
 Charlottesville, VA 22908
 E-mail: dmt6u@virginia.edu

Eric J. Arts
 Department of Microbiology and Immunology
 Schulich School of Medicine and Dentistry
 Western University
 London, ON N6A 3K7
 Canada
 E-mail: earts@uwo.ca