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Performance of a clonal-based HIV-1 tropism phenotypic assay

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Adequate determination of HIV-1 tropism is important in clinical and research settings. Genotypic and phenotypic approaches to evaluate tropism have been described. Phenotypic assays are widely used to determine HIV-1 tropism because of their sensitivity to detect minor CXCR4-using variants (X4). However they cannot differentiate mixed quasi-species of R5 and X4 viruses from dual-tropic viruses. We describe here a clonal-based HIV-1 tropism phenotypic assay. Env-pseudo-typed viruses were produced by co-transfection of the *env* expression plasmid pcDNA3.1/V5HisTOPO and a backbone vector pNL4-3.Luc.E-R- that expresses the entire HIV-1 genome except for *env* and *vpr* in 293T cell cultures. Co-receptor use was tested by infecting U87.CD4.CCR5+ and U87.CD4.CXCR4+ cells in the presence or absence of co-receptor inhibitors, using 10 clones from each sample. The ability of the assay to detect minor variants in a viral population was assessed by mixing X4 and R5 clones using different ratios. Both R5 and X4 minority variants were detected when present at greater than 0.4% in a mixture of envelope populations. This assay can be useful in both clinical and research laboratories.

1. Introduction

Human immunodeficiency virus type 1 (HIV-1) co-receptor tropism refers to the ability of the virus to enter host cells using either the CCR5 or CXCR4 co-receptors named R5 and X4 viruses, respectively (Westby and van der Ryst, 2005). Viruses that can use either CXCR4 or CCR5 are classified as dual-tropic or R5X4 viruses (Berger et al., 1998; Iwamoto et al., 2010; Moore et al., 2004). R5 and X4 viral quasi-species can coexist in a patient's plasma and are classified as mixed-tropic samples (Vandamme et al., 2011). CCR5 inhibitors are increasingly used for HIV treatment and are only active against R5 viruses. Maraviroc (MVC) is the first and only CCR5 inhibitor to be approved by the FDA in 2007 in treatment-experienced patients and in 2009 as a first-line drug in combination with others antiretrovirals. Current HIV treatment guidelines recommend HIV tropism testing when the use of a CCR5 inhibitor is considered (Guidelines, 2013; Rachlis et al., 2010; Vandekerckhove et al., 2011a).

Several genotypic and phenotypic approaches to evaluate tropism have been described (Rose et al., 2009). Genotypic assays use information obtained from sequencing HIV envelope third variable (V3) region to predict viral tropism using various bioinformatic tools and algorithms (Fouchier et al., 1992; Resch and Swanstrom, 2001; Jensen et al., 2003; Pillai et al., 2003; Poveda et al., 2010). Next-generation sequencing provides increased sensitivity to detect minority variants. (Archer et al., 2009), but is still not widely used in clinical practice because of costs and the expertise needed (Poveda et al., 2012). Genotypic methods have shown good correlation with phenotypic tests. Although the predictive value of interpretative algorithms continues to improve, not all the genetic determinants of co-receptor usage reside in the V3 region, therefore affecting their ability to adequately predict tropism (Van Baelen et al., 2007; Vandekerckhove et al., 2011b). Furthermore,

Abbreviations: MVC, maraviroc; R5 viruses, viruses that use CCR5 co-receptor; HIV-1, human immunodeficiency virus 1; X4 viruses, viruses that use co-receptor CXCR4; X4R5/Dual-tropic, viruses that use both co-receptors CCR5 and CXCR4 co-receptors; D/M, mixed virus populations gp120envelope glycoprotein 120; V3, the third variable region; DMEM, Dulbecco modified eagle medium; FCS, fetal calf serum; VL, viral load; Trofile-ES, enhanced version of Trofile; PCR, polymerase chain reaction; RLU, relative light unit; SVM, D/M (dual/mix) populations support vector machines; PSSM, position-specific scoring matrices.

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databases mostly comprise V3 loop sequences from subtypes B and C viruses and most algorithms lack subtype-specific rules (Mulinge et al., 2013).

Phenotypic assays are useful to determine HIV tropism to improve genotypic algorithms and to assess R5 viral resistance to CCR5 antagonists. Phenotypic recombinant assays are the gold standard tests to determine HIV-1 tropism because they have better sensitivity to detect minor X4 variants in various HIV-1 subtypes (Raymond and Izopet, 2012). Currently, the enhanced version of Trofile assay (Trofile-ES) from LabCorp, South San Francisco, CA is one of the most used phenotypic assays for determining co-receptor usage in clinical settings. It can detect X4 virus at 0.3% of virus population (Reeves et al., 2009). It uses *env* sequences obtained from plasma to construct pseudo-typed viruses, which are then used to infect human cell lines that stably express CD4 with either CXCR4 or CCR5 (Whitcomb et al., 2007). This assay has some limitations, such as its high cost, relative long turn-around time, limited availability, and the need for a viral load >1000 HIV RNA copies/ml (Gonzalez-Serna et al., 2010; Poveda and Soriano, 2010; Pfeifer and Lengauer, 2012). Also, it cannot distinguish between truly dual-tropic viruses and mixtures of R5- and X4-tropic viruses (Moyle et al., 2009) and sometimes generates non-reportable results (Genebat et al., 2009; Poveda et al., 2010). There is a need to improve current tropism assays not only to better predict treatment response to CCR5 inhibitors but also to monitor the emergence of drug resistance to this class of antiretrovirals. Clinical resistance to CCR5 inhibitors can evolve through two major pathways. Minority X4 populations present prior to the initiation of therapy can emerge under selective pressure (Westby et al., 2006; Gulick et al., 2007; Landovitz et al., 2008; Demarest et al., 2009; Kitrinos et al., 2009; Tsibris et al., 2009; Cooper et al., 2010; Roche et al., 2013). Alternately, resistant viruses can continue to use CCR5 despite the presence of the inhibitor. As viral resistance to CCR5 inhibitors usually results in cross-resistance to other investigational drugs in the same class (Tilton et al., 2010), a sensitive phenotypic assay to detect minority X4 variants is important to support the development of new co-receptor inhibitors (Wilkin et al., 2007; Landovitz et al., 2008; Hardy et al., 2010; McNicholas et al., 2010, 2011). It is essential to better understand the role of tropism in HIV pathogenesis (Moyle et al., 2005; Hoffmann, 2007; Seclen et al., 2010; Ataher et al., 2012; Ng et al., 2013). We have developed a single-cycle assay based on clonal amplification of HIV-1 *env* gp 160 from HIV-infected individual's' plasma. This assay is sensitive and detects R5 and X4 minority populations in 100% of the mixtures when they are present at a frequency of 0.4% or greater. It can be useful in both clinical and research laboratories.

2. Materials and methods

2.1. Cell lines

Human kidney epithelial 293T cells and U87 cells were cultured in Dulbecco modified Eagle medium (DMEM) (Sigma, St. Louis, MO, USA) supplemented with 10% fetal calf serum (FCS, Invitrogen, Carlsbad, CA, USA), 100 U of penicillin/ml, 0.1 mg of streptomycin/ml. For U87.CD4.CXCR4+ and U87.CD4.CCR5+ cells (NIH AIDS Research and Reference Reagent Program Bethesda, Maryland, USA), 300 μg of G418 (Geneticin; GibcoBRL) per ml was added to the culture. Cells were maintained at 37 °C and 5% CO_2.

2.2. Reference viruses

HIV R5-tropic viruses (BAL and CC1/85), X4 tropic virus (IIIB) and the backbone vector pNL4-3.Luc.E-R- were obtained through the NIH AIDS Research and Reference Reagent Program. Dual tropic virus (85.6) was kindly donated by Dr. Petronela Ancuta.

2.3. Patients

Plasma samples from 31 HIV-1 subtype B chronically infected patients experiencing treatment failure with non-nucleoside reverse transcriptase inhibitor or protease inhibitor based regimens and naïve to CCR5 inhibitors, with viral load (VL) >500 copies/ml (determined with the Roche Amplicor HIV-1 Monitor test), were obtained from subjects attending HIV clinics in Montreal: Clinique L'Actuel, Clinique du Quartier Latin, Centre Hospitalier de l'Université de Montréal. We also used samples 6 harboring viruses from non-B subtypes, 1 AGG subtype, 2 CRF02-A6, 1 AK and 2 AGK subtype. This study was approved by the CRCHUM ethics' review committee.

2.4. Viral RNA isolation

HIV-1 RNA was extracted from patient's plasma and culture supernatants with QIAmp Viral RNA Mini kit (Qiagen, Mississauga, Ontario, Canada). The samples were concentrated 3 times to obtain a final volume of 50 μl and stored at −80 °C for later use.

2.5. Amplification of the HIV envelope gene

Full-length gp160 was amplified. RNA was added as template to a polymerase chain reaction (PCR) master mix containing SuperScript III RT/Platinum Taq HiFi mix, 20 μM of forward and reverse primers: SG3-up: 5′-TACAGTGCAGGGGAAAGAATAATA-GACATAATA-3′ and SG3-lo: 5′-AGACCCAGTACAGGCRARAAGC-3′. Amplification conditions were as follows: an initial 1 h cycle at 50 °C, followed by 2 min cycle at 94 °C for denaturation; 35 cycles with three steps (15 s at 94 °C, 30 s at 55 °C, 5 min at 72 °C); and a final extension cycles for 10 min at 70 °C, 10 min at 4 °C and 10 min at 12 °C. The PCR products were them submitted to a nested PCR using 20 μM forward and reverse primers Env-up: 5′-GTTTCTTTTAGGCATCTCCTATGGCAGGAAGAAG-3′ and Env-lo: 5′-GTTTCTTCCAGTCCCCCCTTTTCTTTTAAAAAG-3′. The amplification conditions in this step were: an initial 2 min cycle at 94 °C, 30 cycles with three steps (15 s at 94 °C, 30 s at 56 °C, 3 min at 72 °C) and a final extension cycles for 7 min at 72 °C, 10 min at 4 °C and 10 min 12 °C. PCR products were migrated on a 1% agarose gel to confirm the presence of a 3000 bp band then purified using QIAprep Spin Miniprep Kit 50 (QIAGEN). Positive (HIV-1 positive sample, a Laboratory adapted BAL virus) and negative controls containing the PCR mixture without the template DNA were used. All constructs were sequenced prior to transfection. Sequencing was performed at Génome Québec (McGill University and Genome Quebec Innovation Centre, Montreal, Quebec, Canada) using 12 primers covering *env*. Sequences were analyzed using Sequencer 4.7 from Gene Codes Corporation software, Ann Arbor, MI and aligned using Clustal W version 1.83 followed by manual alignment editing using BioEdit version 7.0.4.1.

2.6. Cloning of the HIV envelope gene

The Env PCR products of the HIV-1 R5-tropic viruses CC1/85, BAL, X4 tropic virus IIIB, dual tropic virus 85.6 and patients' samples were cloned using an env expression plasmid pcDNA3.1D/V5HisTOPO (Invitrogen, Carlsbad, USA). Env-pseudotyped viruses were produced by co-transfection of the *env* expression plasmid and a backbone vector pNL4-3.Luc.E-R- that expresses the entire HIV-1 genome except for *env* and *vpr*, as described previously (Connor et al., 1996; Singh et al., 2009). The *env* expression plasmid and pNL4-3.Luc.E-R- DNAs