

Assessing Human Immunodeficiency Virus Type 1 Tropism: Comparison of Assays Using Replication-Competent Virus versus Plasma-Derived Pseudotyped Virions[∇]

Noriaki Hosoya,¹ Zhaohui Su,² Timothy Wilkin,³ Roy M. Gulick,³ Charles Flexner,⁴ Michael D. Hughes,² Paul R. Skolnik,⁵ Françoise Giguel,¹ Wayne L. Greaves,⁶ Eoin Coakley,⁷ and Daniel R. Kuritzkes^{1*}

Section of Retroviral Therapeutics, Brigham and Women's Hospital, and Harvard Medical School, Boston, Massachusetts¹; Harvard School of Public Health, Boston, Massachusetts²; Weill Medical College, Cornell University, New York, New York³; Johns Hopkins University, Baltimore, Maryland⁴; Boston University Medical Center, Boston, Massachusetts⁵; Schering-Plough Research Institute, Kenilworth, New Jersey⁶; and Monogram Biosciences, South San Francisco, California⁷

Received 29 March 2009/Returned for modification 6 May 2009/Accepted 26 May 2009

Detection of CXCR4-using human immunodeficiency virus by the Trofile assay was compared to that by assays using virus isolates or replication-competent recombinants. Concordance with the Trofile assay was good, but assays using replicating viruses did not increase substantially the ability to detect the presence of CXCR4-using virus.

Human immunodeficiency virus type 1 (HIV-1) can be assigned to one of three classes based on its ability to utilize the CCR5 and CXCR4 coreceptors: viruses that use CCR5 but not CXCR4 (R5 virus), those that use CXCR4 but not CCR5 (X4 virus), and those that can use either coreceptor (dualtropic virus). HIV-1 also can be classified according to its ability to replicate and induce syncytia in MT-2 cells (8, 12). The use of CXCR4 is a defining feature of syncytium-inducing (SI) viruses in MT-2 cells; most but not all non-SI (NSI) viruses are R5 (1, 13, 16).

Testing to determine coreceptor usage of HIV-1 isolates is essential to identify patients who are suitable candidates for treatment with CCR5 antagonists. The tropism assay (Trofile; Monogram BioSciences, South San Francisco, CA) used in clinical trials of CCR5 antagonists to date is a validated single-cycle assay performed in a Clinical Laboratory Improvement Amendments/College of American Pathologists-certified laboratory; the assay is based on pseudotyped virus and sensitively detects the presence of CXCR4-using virus (14). However, up to 10% of subjects identified as having exclusively R5 virus at screening had evidence of dualtropic or mixed-tropic (D/M) virus at the time treatment with maraviroc or vicriviroc (VCV) was begun (3, 4). To test the hypothesis that the sensitivity of tropism testing could be improved by use of replicating viruses instead of pseudotyped viruses, we compared the results of tropism testing with the Trofile assay to those of assays using replication-competent viruses using clinical samples from AIDS Clinical Trials Group (ACTG) protocol A5211, a phase 2b trial of the investigational CCR5 antagonist VCV (SCH-D; SCH417690; Schering-Plough, Kenilworth, NJ) (4).

(This work was presented in part at the 14th Conference on Retroviruses and Opportunistic Infections, Los Angeles, CA, 25–28 February 2007 [5a].)

Viral tropism by Trofile assay at screening and entry. Eligibility for ACTG A5211 included the exclusive presence of R5 virus as determined by the Trofile assay (4). All subjects provided written informed consent, and all aspects of this study were conducted according to institutional guidelines for research with human subjects. A total of 197 of 391 (50%) subjects had R5 virus at screening, 178 had D/M virus, and 16 had X4 virus (15). Of the 118 subjects with R5 virus who enrolled into the study, 12 (10%) showed D/M virus on repeat testing at entry (4). To assess the relative capacity of D/M viruses from screening and entry samples to use CXCR4 for virus entry, we compared the relative light units (RLU) produced on U87.CD4.CXCR4 cells in the Trofile assay. The D/M viruses identified at screening produced a median of 28,085 RLU, whereas the D/M viruses identified at study entry (from subjects with R5 virus at screening) produced a median of 295 RLU ($P < 0.0001$). This result suggests that the discordant tropism results between screening and entry could be explained by fluctuations of a minority CXCR4-using component of the virus population above and below the limit of assay sensitivity. Alternatively, the predominant virus could have some minimal capacity to use CXCR4 for entry, which likewise fluctuates above and below the assay threshold.

MT-2 cell assay and recombinant virus assays of viral tropism. To determine whether D/M virus could be detected by assay of infectious virus, HIV-1 was isolated from cryopreserved peripheral blood mononuclear cells (PBMCs) obtained at study entry by cocultivation with phytohemagglutinin-stimulated PBMCs from seronegative donors as described previously (5). Infectious HIV-1 was recovered in 55 of 106 cultures (52%) (cryopreserved PBMCs were unavailable from 12 subjects). The viruses were tested for syncytium formation on MT-2 cells by a standard assay (6). Compared to subjects from

* Corresponding author. Mailing address: Brigham and Women's Hospital, 65 Landsdowne Street, Room 449, Cambridge, MA 02139. Phone: (617) 768-8371. Fax: (617) 768-8738. E-mail: dkuritzkes@partners.org.

[∇] Published ahead of print on 3 June 2009.

TABLE 1. Results of coreceptor usage assays^a

Subject	Treatment	Result on study entry				Result on study follow-up with Trofile assay
		Trofile assay	MT-2 cell assay		U87 cell assay with recombinant virus	
			PBMC isolates	Recombinant virus		
1	10 mg VCV	D/M	SI	NSI	D/M	D/M (wk 2)
2	10 mg VCV	D/M	SI	NSI	D/M	X4 (wk 2)
3	15 mg VCV	D/M	SI	NSI	R5	D/M (wk 2)
4	10 mg VCV	D/M	NSI	SI	X4	D/M (wk 2)
5	15 mg VCV	D/M	NSI	NSI	D/M	D/M (wk 2)
6	15 mg VCV	D/M	NSI	NSI	D/M	D/M (wk 2)
7	15 mg VCV	D/M	NSI	NSI	R5	D/M (wk 2)
8	Placebo	D/M	NSI	NSI	D/M	R5
9	5 mg VCV	R5	SI	SI	X4	X4 (wk 8)
10	10 mg VCV	R5	SI	NSI	R5	X4 (wk 2)
11	15 mg VCV	R5	NSI	NSI	D/M	D/M (wk 24)
12	15 mg VCV	R5	NSI	NSI	D/M	D/M (wk 8)
13	15 mg VCV	R5	NSI	SI	D/M	R5
14	5 mg VCV	R5	NSI	NSI	R5	D/M (wk 2)
15	10 mg VCV	R5	NSI			D/M (wk 19)
16	10 mg VCV	R5	NSI			R5
17-48	Various doses of VCV	R5	NSI	NSI	R5	R5
49	Placebo	R5	NSI			R5
50-55	Placebo	R5	NSI	NSI	R5	R5

^a Plasma samples, virus isolates from PBMCs, or recombinant viruses were tested for coreceptor usage or syncytium formation as described in the text. The samples shown as individual line listings tested D/M or X4 in at least one assay or had a missing result due to assay failure. X4, CXCR4-using virus; R5, CCR5-using virus.

whom a virus isolate was not recovered, subjects with a positive virus culture were more likely to be male (96% versus 84%; $P = 0.046$), had somewhat higher plasma HIV-1 RNA levels (4.76 log₁₀ copies/ml versus 4.46 log₁₀ copies/ml; $P = 0.025$), and had lower CD4 cell counts (112 cells/mm³ versus 194 cells/mm³; $P = 0.001$).

Because HIV-1 in plasma is more representative of the actively replicating pool of virus than is virus from PBMCs (9), we also tested coreceptor usage using infectious recombinant viruses that expressed envelope from patient plasma virus in an NL4-3 background. Viral RNA was extracted from 140 µl of plasma using the QIAamp viral RNA minikit (Qiagen, Valencia, CA) and subjected to reverse transcription (RT) and first-round PCR according to the manufacturer's protocol with SuperScript III one-step RT-PCR system with Platinum *Taq* high-fidelity polymerase (Invitrogen, Carlsbad, CA). For an initial RT-PCR a first amplification was carried out using the primers FLenv1.1 (5'-TAGAGCCCTGGAAGCATCCAGGAAG-3') and FLenv1.2 (5'-TTGCTACTTGTGATTGCTCCATGT-3'). One-tenth of the first PCR was subjected to a nested PCR using Platinum *Taq* DNA high-fidelity polymerase (Invitrogen) with primers Lenv2.1 (5'-GATCAAGCTTTAGGCATCTCCTATGGCAGGAAGAAG-3') and FLenv2.2 (5'-AGCTGGATCCGTCTCGAGATACTGCTCCACCC-3'). The cycling conditions were 30 cycles of 30 s at 94°C, 30 s at 56°C, and 4 min at 68°C and a final extension for 5 min at 68°C.

Infectious recombinant viruses were generated by electroporating 2.5 × 10⁶ PM1 cells with 10 µg of the *env* amplicon of interest together with 10 µg of the recombination vector pHIV-1Δ*env*BstEII*nef-hisD*, which carries a full-length infectious molecular clone of HIV-1 NL4-3 from which the entire *env* gene has been deleted (10). Coreceptor usage of recombinant viruses was determined by inoculating 100 µl of viral supernatant onto 0.2 × 10⁶ U87.CD4.CCR5 or U87.CD4.

CXCR4 cells in 24-well plates in a final volume of 1 ml/well. Culture supernatants were sampled every 3 to 4 days for virus production by p24 antigen ELISA. Recombinant viruses were classified as R5, X4, or D/M based on p24 antigen production on U87.CD4.CCR5 or U87.CD4.CXCR4 cells, or both, respectively. Viral replication on U87.CD4.CXCR4 cells was confirmed by inhibiting p24 antigen production with AMD3100, a CXCR4 antagonist (obtained through the AIDS Research and Reference Reagent Program courtesy of Anormed, Inc.). Reconstruction experiments using full-length *env* clones showed the X4 sequences could be detected when present as 1% or more of the population in a background of R5 sequences; D/M sequences could be detected when present in 5% or more of the population.

Among the eight subjects who had D/M virus by the Trofile assay at study entry, 7 (88%) had evidence of CXCR4-using virus at entry by one of the three other assays evaluated, including six who had D/M or X4 using the U87 cell assay, three with SI virus using PBMC isolates, and one with SI virus using recombinant viruses in the MT-2 cell assay (Table 1). Seven of these eight subjects happened to be randomized to receive VCV, including the subject who had NSI virus by the two MT-2 assays and R5 virus by the U87 cell assay. All seven subjects continued to be identified as having D/M or X4 virus at their first on-treatment evaluation (at week 2) by the Trofile assay.

Among 47 subjects who had R5 virus by Trofile at entry, only 5 (11%) had any evidence of SI or D/M or X4 virus at entry by one of the three other assays evaluated (two subjects had no results for the recombinant virus assays). These five subjects included two by each of the two MT-2 cell assays and four by the U87 cell assay. All five of these subjects happened to be randomized to receive VCV, and four of the five subsequently were identified as having X4 or D/M virus by Trofile while on VCV. There were only two subjects with R5 virus at entry by

Trofile in whom CXCR4-using virus was not detected by another assay prior to developing D/M virus while receiving VCV (although one of these two did not have results from the recombinant virus assays).

Conclusions. In this study we compared the ability of four assays to detect the presence of CXCR4-using HIV-1 in subjects commencing treatment with the CCR5 antagonist VCV. Assays using virus isolates or replication-competent recombinant viruses showed good concordance with the Trofile assay overall. Contrary to our expectations, the multiple-cycle assays did not increase the ability to detect the presence of CXCR4-using virus at study entry in samples from subjects in whom D/M or X4 virus eventually emerged during VCV treatment.

Limitations of the study include the modest sample size due to low recovery of HIV-1 isolates from cryopreserved PBMCs, which is consistent with results of previous studies (7); higher isolation rates can be achieved using fresh PBMCs (2). Because virus isolation was most often successful in subjects with higher plasma HIV-1 RNA levels and lower CD4 cell counts, different results might have been obtained if isolates from subjects with lower plasma HIV-1 RNA levels and higher CD4 counts were available. Different results might also have been obtained if the samples from the entire screening population (rather than the enrolled population) were available for study.

The emergence of D/M or X4 virus in VCV recipients who had exclusively R5 or NSI virus in the assays studied here underscores the need for more sensitive assays that are better able to detect the preexisting CXCR4-using minority variants in the virus population. An enhanced Trofile assay with improved sensitivity has replaced the original assay for use in clinical practice (11).

We gratefully acknowledge the efforts of the ACTG A5211 protocol team, the investigators and staff at the participating AIDS Clinical Trials Units, and the study subjects. We thank Schering-Plough for providing the study drug, and Monogram Biosciences for performing the Trofile assays for this study. The following reagents were obtained from the NIH AIDS Research and Reference Reagent Program: MT-2 cells from D. Richman; PM1 cells from M. Reitz; U87.CD4.CCR5 and U87.CD4.CXCR4 cells from H. Deng and D. R. Littman; and interleukin-2 from M. Gately.

This work was supported by NIH grants AI068636 (ACTG), AI051966, AI055357, AI069419, AI069472, RR024996, and RR16482; a subcontract from AI068636 to the Virology Support Laboratory at Massachusetts General Hospital; the Harvard University Center for AIDS Research (AI060354); and the Japanese Foundation for AIDS Prevention (JFAP).

REFERENCES

- Björndal, Å., H. Deng, M. Jansson, J. R. Fiore, C. Colognesi, A. Karlsson, J. Albert, G. Scarlatti, D. R. Littman, and E. M. Fenyo. 1997. Coreceptor usage of primary human immunodeficiency virus type 1 isolates varies according to biological phenotype. *J. Virol.* **71**:7478–7487.
- Demeter, L. M., R. J. Bosch, R. W. Coombs, S. Fiscus, J. Bremer, V. A. Johnson, A. Erice, J. B. Jackson, S. A. Spector, K. M. Squires, M. A. Fischl, M. D. Hughes, and S. M. Hammer. 2002. Detection of replication-competent human immunodeficiency virus type 1 (HIV-1) in cultures from patients with levels of HIV-1 RNA in plasma suppressed to less than 500 or 50 copies per milliliter. *J. Clin. Microbiol.* **40**:2089–2094.
- Fatkenheuer, G., M. Nelson, A. Lazzarin, I. Konourina, A. I. Hoepelman, H. Lampiris, B. Hirschel, P. Tebas, F. Raffi, B. Trottier, N. Bellos, M. Saag, D. A. Cooper, M. Westby, M. Tawadrous, J. F. Sullivan, C. Ridgway, M. W. Dunne, S. Felstead, H. Mayer, and E. van der Ryst. 2008. Subgroup analyses of maraviroc in previously treated R5 HIV-1 infection. *N. Engl. J. Med.* **359**:1442–1455.
- Gulick, R. M., Z. Su, C. Flexner, M. D. Hughes, P. R. Skolnik, T. J. Wilkin, R. Gross, A. Krambrink, E. Coakley, W. L. Greaves, A. Zolopa, R. Reichman, C. Godfrey, M. Hirsch, D. R. Kuritzkes, et al. 2007. Phase 2 study of the safety and efficacy of vicriviroc, a CCR5 inhibitor, in HIV-1-infected, treatment-experienced patients. *J. Infect. Dis.* **196**:304–312.
- Hollinger, F. B., J. W. Bremer, L. E. Myers, J. W. M. Gold, L. McQuay, and The NIH/NIAD/DAIDS/ACTG Virology Laboratories. 1992. Standardization of sensitive human immunodeficiency virus coculture procedures and establishment of a multicenter quality assurance program for the AIDS Clinical Trials Group. *J. Clin. Microbiol.* **30**:1787–1794.
- Hosoya, N., Z. Su, T. Wilkin, R. M. Gulick, C. Flexner, M. Hughes, P. R. Skolnik, W. L. Greaves, E. Coakley, and D. R. Kuritzkes. 2007. 14th Conf. Retrovir. Opportunistic Infect., abstr. 181b.
- Japour, A. J., S. A. Fiscus, J.-M. Arduino, D. L. Mayers, P. S. Reichelderfer, and D. R. Kuritzkes. 1994. Standardized microtiter assay for the determination of syncytium-inducing phenotypes of clinical human immunodeficiency virus type 1 isolates. *J. Clin. Microbiol.* **32**:2291–2294.
- Japour, A. J., S. Welles, R. T. D'Aquila, V. A. Johnson, D. D. Richman, R. W. Coombs, P. S. Reichelderfer, J. O. Kahn, C. S. Crumpacker, and D. R. Kuritzkes. 1995. Prevalence and clinical significance of zidovudine resistance mutations in human immunodeficiency virus isolated from patients after long-term zidovudine treatment. *J. Infect. Dis.* **171**:1172–1179.
- Koot, M., A. H. V. Vos, R. P. M. Keet, R. E. Y. de Goede, M. W. Dercksen, F. G. Terpstra, R. A. Coutinho, F. Miedema, and M. Tersmette. 1992. HIV-1 biological phenotype in long-term infected individuals evaluated with an MT-2 cocultivation assay. *AIDS* **6**:49–54.
- Kozal, M. J., R. W. Shafer, M. A. Winters, D. A. Katzenstein, and T. C. Merigan. 1993. A mutation in human immunodeficiency virus reverse transcriptase and decline in CD4 lymphocyte numbers in long-term zidovudine recipients. *J. Infect. Dis.* **167**:526–532.
- Lu, J., P. Sista, F. Giguel, M. Greenberg, and D. R. Kuritzkes. 2004. Relative replicative fitness of human immunodeficiency virus type 1 mutants resistant to enfuvirtide (T-20). *J. Virol.* **78**:4628–4637.
- Reeves, J., D. Han, T. Wilkin, T. Wrin, D. Kuritzkes, C. Petropoulos, J. Whitcomb, N. Parkin, R. Gulick, and E. Coakley. 2008. An enhanced version of the Trofile HIV co-receptor tropism assay predicts emergence of CXCR4 use in ACTG5211 vicriviroc trial samples, abstr. 869. Abstr. 15th Conf. Retrovir. Opportunistic Infect.
- Schuitmaker, H., M. Koot, N. A. Kootstra, M. W. Dercksen, R. E. Y. de Goede, R. P. van Steenwijk, J. M. A. Lange, J. K. M. E. Schattnerkerk, F. Miedema, and M. Tersmette. 1992. Biological phenotype of human immunodeficiency virus type 1 clones at different stages of infection: progression of disease is associated with a shift from monocytotropic to T-cell-tropic virus populations. *J. Virol.* **66**:1354–1360.
- Simmons, G., D. Wilkinson, J. D. Reeves, M. T. Dittmar, S. Beddows, J. Weber, G. Carnegie, U. Desselberger, P. W. Gray, R. A. Weiss, and P. R. Clapham. 1996. Primary, syncytium-inducing human immunodeficiency virus type 1 isolates are dual-tropic and most can use either Lestr or CCR5 as coreceptors for virus entry. *J. Virol.* **70**:8355–8360.
- Whitcomb, J. M., W. Huang, S. Fransen, K. Limoli, J. Toma, T. Wrin, C. Chappay, L. D. B. Kiss, E. E. Paxinos, and C. J. Petropoulos. 2007. Development and characterization of a novel single-cycle recombinant-virus assay to determine human immunodeficiency virus type 1 coreceptor tropism. *Antimicrob. Agents Chemother.* **51**:566–575.
- Wilkin, T. J., Z. Su, D. R. Kuritzkes, M. Hughes, C. Flexner, R. Gross, E. Coakley, W. Greaves, C. Godfrey, P. R. Skolnik, J. Timponi, B. Rodriguez, R. M. Gulick, et al. 2007. Chemokine coreceptor tropism among antiretroviral-experienced patients screening for a clinical trial of a CCR5 inhibitor. *Clin. Infect. Dis.* **44**:591–595.
- Zhang, L., T. He, Y. Huang, Z. Chen, Y. Guo, S. Wu, K. J. Kunstman, R. C. Brown, J. P. Phair, A. U. Neumann, D. D. Ho, and S. M. Wolinsky. 1998. Chemokine coreceptor usage by diverse primary isolates of human immunodeficiency virus type 1. *J. Virol.* **72**:9307–9312.