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Comparison of Human Immunodeficiency Virus Type 1 Tropism Profiles in Clinical Samples by the Trofile and MT-2 Assays[▽]

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The recent availability of CCR5 antagonists as anti-human immunodeficiency virus (anti-HIV) therapeutics has highlighted the need to accurately identify CXCR4-using variants in patient samples when use of this new drug class is considered. The Trofile assay (Monogram Biosciences) has become the method that is the most widely used to define tropism in the clinic prior to the use of a CCR5 antagonist. By comparison, the MT-2 assay has been used since early in the HIV epidemic to define tropism in clinical specimens. Given that there are few data from direct comparisons of these two assays, we evaluated the performance of the plasma-based Trofile assay and the peripheral blood mononuclear cell (PBMC)-based MT-2 assay for the detection of CXCR4 use in defining the tropism of HIV isolates derived from clinical samples. The various samples used for this comparison were derived from participants of the Amsterdam Cohort Studies on HIV infection and AIDS who underwent consecutive MT-2 assay testing of their PBMCs at approximately 3-month intervals. This unique sample set was specifically selected because consecutive MT-2 assays had demonstrated a shift from negative to positive in PBMCs, reflecting the first emergence of CXCR4-using virus in PBMCs above the level of detection of the assay in these individuals. Trofile testing was performed with clonal HIV type 1 (HIV-1) variants ($n = 21$), MT-2 cell culture-derived cells ($n = 20$) and supernatants ($n = 42$), and plasma samples ($n = 76$). Among the clonal HIV-1 variants and MT-2 cell culture-derived samples, the results of the Trofile and MT-2 assays demonstrated a high degree of concordance (95% to 98%). Among consecutive plasma samples, detection of CXCR4-using virus was at or before the time of first detection by the MT-2 assay in 5/10 patients by the original Trofile assay and in 9/10 patients by the enhanced-sensitivity Trofile assay. Differences in the time to the first detection of CXCR4 use between the MT-2 assay (PBMCs) and the original Trofile assay (plasma) were greatly reduced by the enhanced-sensitivity Trofile assay, suggesting that sensitivity for the detection of minor CXCR4-using variants may be a more important determinant of discordant findings than compartmentalization. The similarities in performance of the enhanced-sensitivity Trofile and MT-2 assays suggest that either may be an appropriate methodology to define tropism in patient specimens.

The outcome of exposure to human immunodeficiency virus type 1 (HIV-1) varies greatly between individuals. One of the factors determining this variability in outcome is the cellular tropism or viral phenotype (7, 23), as the pathogenesis of HIV-1 is critically influenced by the cell types that the virus is capable of infecting. HIV-1 requires two cellular receptors for entry: CD4 and one of a family of chemokine receptors (coreceptor). In vivo, the major coreceptors used by HIV-1 are CCR5 (1, 13, 14) and CXCR4 (15). Individual viruses are classified on the basis of their ability to use CCR5 (R5 variants) or CXCR4 (X4 variants), or both (R5X4 or dual variants) (2). Viral populations demonstrating the use of both receptors are designated dual/mixed (D/M), as they may contain any mixture of these three types (43). Before the identification of HIV coreceptors, viruses were classified on the basis of their ability to infect T-cell lines and/or macrophages and/or on the basis of their ability to induce syncytia (multinucleated giant cells) in MT-2 cells (16, 36) which do not express CCR5 (11). Thus, viruses that do not infect MT-2 cells are non-syncytium inducing and R5, while viruses that do infect MT-2 cells are syncytium inducing and either X4 or R5X4 (4). Determinants that govern coreceptor use have been mapped to the envelope gene, especially to the variable regions, and in particular the third variable (V3) region, the V3 loop (12, 17).

Of note, the ability to induce syncytia in MT-2 cells reflects the viral coreceptor use and not necessarily the enhanced cytopathogenicity of X4 virus per se, as R5 virus can at least be equally cytopathogenic to its target cells (18, 26, 33). The differential pathogenicity of R5 and X4 viruses in vivo mainly lies in the coreceptor expression patterns of the host cells. CXCR4 is expressed on many more $CD4^+$ cells in the body (including hematopoietic progenitor cells, thymocytes, naïve T cells, and monocytes) than CCR5, which is mostly found on memory T cells and macrophages (3, 6). Thus, CXCR4 use potentially allows the virus access to a large and critical pool of target cells affecting T-cell ontogeny, a fact that may be relevant to the accelerated $CD4^+$ T-cell decline associated with the emergence of CXCR4-using virus (5). Therefore, detection of

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the emergence of CXCR4-using virus has potential value for predicting pathogenesis, monitoring disease progression, and making treatment decisions. Moreover, the detection of CXCR4-using variants is required for the optimal use of the recently available CCR5 antagonists as HIV therapeutics, as the activity of CCR5 antagonists in patients is attenuated in the presence of CXCR4-using HIV (27).

Traditionally, the MT-2 assay has served as a tool for the determination of tropism on the basis of the expression of CXCR4 (and not CCR5) on the cell surface. There are a wealth of data correlating the detection of CXCR4-using virus by the MT-2 assay and accelerated disease progression in treatment-naïve individuals (7, 8, 23, 29, 32). Two widely used versions of the MT-2 assay exist: one in which the patient's cells are directly cocultured with the MT-2 cells (25) and another in which virus stocks are first generated from the patient's cells by coculture with seronegative phytohemagglutinin-stimulated peripheral blood mononuclear cells (PBMCs) (22). The latter approach may limit the sensitivity of the assay, as MT-2 cells are more sensitive to infection by CXCR4-using virus than PBMCs. Moreover, PBMC passaging of patient samples prior to MT-2 inoculation has the potential to alter the relative proportions of viral subpopulations present in the original sample (42). Although the MT-2 assay is not technically challenging, the need for viable patient cells (either fresh or cryopreserved) and biosafety level 3 laboratories has limited its widespread implementation.

By comparison, the Trofile assay (Monogram Biosciences) was utilized in the MOTIVATE trials, which led to the approval of maraviroc, the first in the class of CCR5 antagonists (19). This assay, specifically adapted for high-throughput testing of patient samples, is available in 35 countries; and with more than 54,000 samples tested to date, the assay is the only clinically validated test available for the screening of patients considering CCR5 antagonist therapy (9, 19, 21, 28, 30, 44). The Trofile assay evaluates the coreceptor use of recombinant luciferase-reporter viruses pseudotyped with a population (or clones) of patient-derived HIV envelopes in a single-cycle infection assay. The original version of the assay was validated to detect low-level X4 or R5 variants with 100% sensitivity when those variants comprised 10% of a mixed HIV envelope population and with 85% sensitivity when those variants comprised 5% of a population (43). Clinical trial data indicated that an improved sensitivity for the detection of low-level CXCR4-using variants might improve the selection of patients for CCR5 antagonist therapy (19, 20). Therefore, an enhanced version of the Trofile assay with an approximately 30-fold increased sensitivity for the detection of low-level X4 variants was developed and validated to detect low-level X4 variants with 100% sensitivity when the variants comprised as little as 0.3% of a mixed envelope population (31). Retrospective analyses of two studies evaluating CCR5 antagonist activity among individuals with R5 tropism defined by the original Trofile assay demonstrated improved outcomes among those with R5 tropism defined by the enhanced-sensitivity Trofile assay (34, 35). The assay with enhanced sensitivity replaced the original Trofile assay in the clinic in June 2008.

In this study we explored the performance of the Trofile assay for the detection of HIV-1 tropism profiles in clinical specimens derived from individuals in whom consecutive MT-2 assays at 3-month intervals had detected the emergence of CXCR4-using virus in PBMCs for the first time. The samples tested were plasma, clonal HIV-1 variants, and cells and cell-free supernatants derived from MT-2 cell cultures. Testing of clonal HIV-1 variants and samples derived from MT-2 cell cultures allowed a comparison of the tropism characterization of homogeneous viral populations. Testing of consecutive patient samples allowed interrogation of the performance of both the MT-2 assay (with PBMCs) and the Trofile assay (with plasma) near the limits of detection for CXCR4-using variants.

MATERIALS AND METHODS

Cohort. The samples evaluated (PBMC and plasma samples obtained from regular blood draws) were derived from 10 participants in the Amsterdam Cohort Studies on HIV Infection and AIDS (ACS) who originally underwent tropism testing by the MT-2 assay at approximately 3-month intervals. The samples selected for this study were from consecutive time points around the time of first detection of CXCR4-using HIV by the MT-2 assay. The patients were homosexual men who were seropositive on entry into the ACS between 1985 and 1995. None experienced an AIDS-defining illness during the time of sampling for this study. Each patient had a documented date of first detection of CXCR4-using virus by the MT-2 assay which was preceded by at least two negative MT-2 assay scores in the preceding 6 months. At the time of the first positive MT-2 assay result (time zero), the participants were aged 41 years, on average (age range, 25 to 57 years), and had average CD4 counts of 491 $CD4^+$ T cells/μl (range, 210 to 890 $CD4^+$ T cells/μl). Plasma HIV-1 RNA testing was not routinely available at the time that these plasma samples were drawn. Seven patients were not receiving antiretroviral therapy at the time of sampling, while three patients (patients 13885, 13907, and 13908) received zidovudine with or without zalcitabine at the last one or two time points.

The ACS has been conducted in accordance with the ethical principles set out in the Declaration of Helsinki, and written informed consent was obtained prior to data collection. The study was approved by the Academic Medical Center institutional medical ethics committee.

Samples. In order to compare tropism characterization in homogeneous viral populations, testing of clonal HIV-1 variants and samples derived from MT-2 cell cultures was performed. To this end, two approaches were taken. First, 21 clonal HIV-1 variants with tropism profiles well defined by the MT-2 assay were tested by the original Trofile assay. The clonal HIV-1 variants were obtained by limiting-dilution biological cloning from two ACS patients (patients 19792 and 19961), as described previously (39–41). Second, cryopreserved PBMCs from 4 of the 10 patients studied here underwent repeat testing by the MT-2 assay at each of the time points surrounding the original positive determination by the MT-2 assay. From these assays, both cell-free supernatants and cells were harvested 28 days after the initiation of the PBMC and MT-2 cell coculture and were tested by the original Trofile assay.

In order to interrogate the performance of the assays near the limits of detection for CXCR4-using isolates, testing of consecutive plasma samples was performed. The plasma samples were tested by the original Trofile assay and also by the enhanced version of the Trofile assay. In all cases, blinded cryopreserved samples were sent on dry ice to Monogram Biosciences for testing by the Trofile assay.

MT-2 assay. In the MT-2 assay, duplicate cultures of 1×10^6 patient PBMCs were cocultured with 1.6×10^6 MT-2 cells (a kind gift from Brendan Larder, United Kingdom) in 5 ml of Iscove's modified Dulbecco's medium supplemented with fetal bovine serum (10%), penicillin (100 U/ml), and streptomycin (100 μg/ml) for 3 to 4 weeks with biweekly light microscopy for detection of the typical cytopathic effect (CPE) associated with CXCR4-using virus infection and for passaging (25). Testing by the MT-2 assay was originally performed in real time (i.e., with fresh samples tested immediately after the blood draw) with samples harvested during the period from 1992 to 2000. At that time, MT-2 assay cultures were maintained for 3 weeks, at which time the culture supernatant was also tested for viral *gag* p24 production by an in-house p24 enzyme-linked immunosorbent assay (37).

For four patients in this study, the MT-2 assays were repeated in 2006 with cryopreserved PBMCs and by use of a slight modification of the methodology described above. These cultures were maintained for 4 weeks, and testing for p24 production was not performed. An in-house validation study performed in 2003 showed that the additional week of culture was more sensitive and obviated the need for testing of p24 production (data not shown). As a positive control,

duplicate cultures were inoculated with 25 HIV-1 IIIB-infected H9 cells. Duplicate cultures of uninfected MT-2 cells served as negative controls. If neither of the patient's two duplicate cultures showed a CPE, then the sample was scored negative. If one or both cultures showed a CPE, then the sample was scored positive. The clonal HIV-1 variants described above were typed by the MT-2 assay by coculturing 1×10^6 phytohemagglutinin-stimulated PBMCs infected with the virus with 1×10^6 MT-2 cells for 1 week and screening for a CPE at days 3, 4, and 7. The sensitivity of the MT-2 assay was determined in previous studies by comparing the results of limiting-dilution biological cloning with those of the MT-2 assay for 55 different cryopreserved PBMC samples obtained from 12 different ACS participants (an average of 5 samples per patient) and is one productively infected cell per 10^6 PBMCs (data not shown).

Trofile assay. In common with the MT-2 assay, the Trofile assay (Monogram Biosciences) is also a cell-based/phenotypic method for the determination of viral tropism. Distinct from the MT-2 assay, the Trofile assay utilizes recombinant virus derived from patient plasma and employs test cell lines that permit the discrimination of CXCR4 and CCR5 use. Briefly, the Trofile assay is a single-cycle recombinant virus assay in which a population of full-length patient-derived *env* genes is amplified by reverse transcription-PCR and cloned into an Env expression vector library that is used to generate luciferase-reporter pseudoviruses (43). These are subsequently used to challenge U87 target cells expressing CD4 and either CCR5 or CXCR4 coreceptors in a 96-well plate format. Infection is determined by assaying for luciferase activity in the presence and the absence of specific inhibitors of the CCR5 or CXCR4 coreceptor as specificity controls; and viral tropism is reported as R5, X4, or D/M. Two versions of the Trofile assay have been developed: the original Trofile assay and the enhanced-sensitivity version of the assay, which has an improved ability to detect CXCR4-using variants present at low levels. The methodologies for both the original and the enhanced-sensitivity Trofile assays are fundamentally the same except that enhanced detection of low levels of CXCR4 minor variants was achieved by optimizing the virus production and infection conditions. The means of optimization included the production of virus in a smaller volume of medium to effectively concentrate the virus stock and the use of a larger volume of virus stock for the infection step. Compared to the original Trofile assay, the enhanced-sensitivity assay has an approximately 30-fold increased sensitivity for the detection of X4 variants present at low levels and has been found to detect 0.3% X4 variants in a mixed envelope population with 100% sensitivity in assay validation experiments (31). As noted above, the version of the assay with enhanced sensitivity replaced the original assay in the clinic in June 2008. Detailed procedures and assay performance characteristics for the Trofile assay are reported elsewhere (31, 43).

RESULTS

Concordant phenotyping results for clonal HIV-1 variants and MT-2 coculture supernatants and cells. In order to compare the characterization of tropism in homogeneous viral populations, testing of clonal HIV-1 variants and samples derived from MT-2 cell cultures was performed. Among the clones, nine were scored negative by the MT-2 assay and 12 were scored positive. As can be seen from Table 1, the Trofile and MT-2 assay tropism calls were concordant for 20/21 clones (95%); clone 17 was defined as R5X4 by the Trofile assay and negative by the MT-2 assay.

Cell and supernatant samples from four patients undergoing repeat MT-2 assay testing were also tested by the Trofile assay. Since MT-2 cultures are performed in duplicate for each sample, materials from two parallel cultures were available for testing by the Trofile assay for each time point. Table 2 depicts the MT-2 and Trofile assay results for the duplicate MT-2 cell culture-derived supernatants. For paired cell and supernatant samples, the Trofile assay results were fully concordant. The results of the MT-2 and Trofile assays were concordant for 41/42 (98%) supernatant samples and 20/21 (95%) cell samples. Discordance was seen for one sample, which was negative by the MT-2 assay but for which both cells and supernatant were D/M by the Trofile assay. Thus, we observed an overall high degree of concordance for clonal HIV-1 variants and for both cells and culture supernatants (≥95%).

TABLE 1. Comparison of MT-2 and Trofile assay results for 21 clonal HIV-1 variants

Patient identifier	Clone identifier	Result by[a]:	
		MT-2 assay	Trofile assay
19961	1	−	R5
	2	−	R5
	3	+	R5X4
	4	−	R5
	5	+	R5X4
	6	+	R5X4
	7	+	R5X4
	8	−	R5
	9	+	R5X4
	10	+	R5X4
	11	−	R5
	12	+	R5X4
19792	13	−	R5
	14	+	R5X4
	15	−	R5
	16	+	R5X4
	17	−	R5X4
	18	+	R5X4
	19	−	R5
	20	+	R5X4
	21	+	R5X4

[a] −, negative by MT-2 culture; +, positive by MT-2 culture; R5, CCR5 using; R5X4, capable of using both CCR5 and CXCR4.

Detection of CXCR4-using variants in longitudinal PBMC and plasma samples. For 10 patients, seven to nine consecutive plasma samples obtained at times surrounding the time of the documented emergence of CXCR4-using variants in PBMCs by the MT-2 assay were tested by the original Trofile assay. Detailed results for each patient sample are shown in Table 3. For patient 13907, the results of all assays matched for each sample tested. In contrast, for patients 13904, 13940, 13951, 13988, and 13993, the original Trofile assay first detected CXCR4-using variants 3 to 11 months later than the MT-2 assay. Conversely, for patient 13908, CXCR4-using variants were detected 3 months prior to their detection by the MT-2 assay. The MT-2 assay had a technical failure for one PBMC sample (patient 13904, visit 28 [V28]), while the original Trofile assay gave no results for four plasma samples (patient 13885, V9 and V10; patient 12912, V11; and patient 13988, V24), possibly due to low plasma viral loads. Overall, a shift from R5 to D/M virus was observed in plasma samples from all 10 patients by the original Trofile assay; however, the times of first detection differed (Table 4). Comparing the time of first detection of CXCR4-using HIV, the Trofile assay defined D/M tropism at ($n = 4$) or before ($n = 1$) the first positive MT-2 culture result for 5/10 patients. For the remaining five patients, D/M HIV variants were detected 3, 3, 6, 7, and 11 months after first positive MT-2 culture result, respectively.

To explore whether this difference in the time point of the switch determined by the two assays was the result of differences in assay sensitivity or sample type (PBMCs versus plasma), the samples were retested by the recently introduced version of the Trofile assay with enhanced sensitivity (31, 38).

TABLE 2. Comparison of MT-2 and Trofile assay results for MT-2 culture supernatants and cells[a]

Patient identifier	Visit no.	Time (mo)	Result for:				
			Flask 1			Flask 2	
			MT-2 assay	Trofile assay		MT-2 assay	Trofile assay supernatant
				Supernatant	Cells		
13993	35	0	+	D/M	D/M	−	−
	36	3	+	D/M	D/M	+	D/M
	37	6	+	D/M	D/M	+	D/M
	38	9	+	D/M	D/M	+	D/M
	39	12	+	D/M	D/M	+	D/M
13988	19	0	−	−	−	−	−
	20	3	−	−	−	−	−
	21	6	−	−	−	−	−
	22	9	−	−	−	−	−
	23	12	+	D/M	D/M	+	D/M
	24	15	+	D/M	D/M	+	D/M
13951	20	0	−	−	−	−	−
	21	3	+	D/M	D/M	−	−
	22	6	+	D/M	D/M	+	D/M
	23	9	+	D/M	D/M	−	−
	24	12	+	D/M	D/M	+	D/M
13940	17	0	−	D/M	D/M	−	−
	18	3	−	−	−	+	D/M
	19	5	+	D/M	D/M	+	D/M
	20	9	+	D/M	D/M	+	D/M
	21	12	+	D/M	D/M	+	D/M

[a] Time, time from first visit tested in this study; flask 1, first flask of the two duplicate cultures of the MT-2 assay; flask 2, second flask of the two duplicate cultures of the MT-2 assay; MT-2 assay, results of the MT-2 assay performed with cryopreserved patient PBMCs; Trofile assay, results of the original Trofile assay performed with the supernatant or cells obtained at the end of the MT-2 assay (the cells from flask 2 were not tested); −, negative MT-2 culture or negative Trofile result; +, positive MT-2 culture result; D/M, dual or mixed tropism. When MT-2 cultures are scored negative, no virus was presumed to be present in the culture supernatant, and hence, the Trofile assay result was negative (i.e., no replication was detected in the CCR5- and the CXCR4-expressing cell cultures).

The 12 samples that were defined as R5 by the original Trofile assay were reclassified as D/M by the enhanced-sensitivity Trofile assay, including 4 samples that were scored negative by the MT-2 assay (Tables 3 and 4). As a result, the enhanced-sensitivity Trofile assay detected CXCR4-using HIV-1 at ($n = 6$) or before ($n = 3$) the first positive MT-2 culture result for 9/10 patients; it is intriguing, however, that for patient 13988, CXCR4-using variants were still detected only 11 months after their first detection by MT-2 culture.

In samples from the four patients who underwent repeat testing by the MT-2 assay, as described above (Tables 2 and 3), the timing of the first positive MT-2 culture result differed from that of the original MT-2 determination by −6, −3, −2, and +3 months.

DISCUSSION

Coreceptor use of HIV-1 is an important determinant of disease progression in the natural course of infection (23). The availability of CCR5 antagonists in the clinic has emphasized the role of coreceptor profiling in the setting of antiretroviral treatment. Phenotypic assays for the determination of coreceptor use include the PBMC-based MT-2 assay (25) and the plasma-based Trofile assay (43). Most coreceptor use data for longitudinal samples obtained during the natural course of disease have been obtained by the MT-2 assay, and these data robustly define the association between the emergence of CXCR4-using virus and accelerated disease progression (7, 8, 23, 29, 32). On the other hand, most cross-sectional tropism data for clinical samples have been obtained by the Trofile assay (9, 19, 21, 28, 30, 44); in addition, the Trofile assay has been used to clinically validate the efficacies of CCR5 antagonists. In this study, we compared the ability of both assays to detect CXCR4-using virus in samples obtained at about the time of first detection of CXCR4-using virus by the MT-2 cell assay. The samples selected comprised two groups; the first group was selected with the intent of exploring the categorization of both assays for homogeneous viral populations and consisted of clonal HIV-1 variants and virus from MT-2 cell culture supernatants and cells. A second group was used to explore the timing of detection of CXCR4-using HIV in plasma by the Trofile assay and consisted of a unique sample set specifically selected because consecutive MT-2 assays had demonstrated a shift from negative to positive when PBMCs were tested, reflecting the first emergence of CXCR4-using virus in PBMCs above the level of detection of the assay in these individuals.

The results for clonal HIV-1 variants and for MT-2 culture supernatants/cells were highly concordant between the two assays, suggesting that the levels of CXCR4-using variants in these cultured specimens are high enough to easily exceed the detection limit of both assays.

For the 10 patients whose consecutive plasma and PBMC samples were tested, both assays were able to detect the

TABLE 3. Detection of CXCR4-using virus in consecutive PBMCs and plasma samples from 10 homosexual male participants in ACS by MT-2 or Trofile assay[a]

Participant	ACS visit no.	Time (mo)	Result by:			
			Original MT-2 assay	Repeat MT-2 assay	Original Trofile assay	Enhanced-sensitivity Trofile assay
13993	35	0	−	+	R5	R5
	36	3	−	+	R5	R5
	37	6	+	+	R5	D/M
	38	9	+	+	D/M	D/M
	39	12	−	+	D/M	D/M
	40	15	−		D/M	D/M
	41	18	−		D/M	D/M
	42	21	+		D/M	D/M
	43	34	+		D/M	D/M
13988	19	0	−	−	R5	R5
	20	3	−	−	R5	R5
	21	6	−	−	R5	R5
	22	9	+	−	R5	R5
	23	12	+	+		
	24	15	+	+	Fail	
	25	18	+		R5	R5
	26	20	+		D/M	D/M
	27	23	+		D/M	D/M
	28	27	+		R5	R5
13951	20	0	−	−	R5	R5
	21	3	−	+	R5	R5
	22	6	+	+	R5	D/M
	23	9	+	+	R5	R5
	24	12	+	+	D/M	D/M
	25	15	+		R5	D/M
	27	19	+		R5	D/M
13940	17	0	−	−	R5	D/M
	18	3	−	+	R5	D/M
	19	5	+	+	R5	D/M
	20	9	+	+	R5	D/M
	21	12	+	+	D/M	D/M
	22	15	+		R5	D/M
	23	18	+		D/M	D/M
	25	21	+		D/M	D/M
13912	10	0	−		R5	R5
	11	3	−		Fail	
	12	6	+		D/M	D/M
	13	9	+		D/M	D/M
	14	12	+		R5	R5
	15	15	+		D/M	
	16	18	+		D/M	
13908	8	0	−		R5	R5
	9	4	−		D/M	D/M
	10	7	+		D/M	D/M
	11	10	+		D/M	D/M
	14	14	+		D/M	D/M
	26	27	+		D/M	D/M
	41	43	+		D/M	D/M
13907	7	0	−		R5	R5
	8	2	−		R5	R5
	9	5	+		D/M	D/M
	10	8	+		D/M	D/M
	11	11	+		D/M	D/M
	15	18	+		D/M	D/M
	25	26	+		D/M	D/M
	32	33	+		D/M	D/M

Continued on following page

TABLE 3—*Continued*

Participant	ACS visit no.	Time (mo)	Result by:			
			Original MT-2 assay	Repeat MT-2 assay	Original Trofile assay	Enhanced-sensitivity Trofile assay
13904	19	0	−		R5	R5
	20	1	−		R5	R5
	22	3	+		R5	D/M
	23	4	+		R5	R5
	25	6	+		D/M	D/M
	26	6	+		D/M	D/M
	28	8	Fail		D/M	D/M
13885	9	0	−		Fail	
	10	3	−		Fail	
	11	6	+		D/M	D/M
	12	9	+		D/M	D/M
	13	12	+		D/M	D/M
	16	21	+		D/M	D/M
	29	39			D/M	D/M
13845	2	0	−		R5	D/M
	3	3	−		R5	D/M
	4	6	+		D/M	D/M
	5	9	+		D/M	D/M
	6	12	+		D/M	D/M
	7	15	+		D/M	D/M
	9	21			D/M	D/M

[a] Time, time from the first visit tested in this study; original MT-2 assay, results of the MT-2 assay performed with fresh patient PBMCs in real time; repeat MT-2 assay, results of the MT-2 assay performed with cryopreserved patient PBMCs from the same time points in 2006; −, negative MT-2 culture result; +, positive MT-2 culture result; R5, CCR5 using; Fail, assay failure; D/M, dual or mixed tropism.

emergence of CXCR4-using variants. However, for 5/10 patients the original Trofile assay detected this at least 3 months later than the MT-2 assay. This could reflect differences between the plasma virus and PBMC virus populations. Indeed, CXCR4-using variants may emerge later in plasma than they do in PBMCs. Alternatively, the detection limit of the original Trofile assay might not be low enough to detect the CXCR4-using variants as they are first emerging. To test the latter hypothesis, we also used a version of the Trofile assay that has an approximately 30-fold increased sensitivity for the detection of CXCR4-using variants compared with that of the original Trofile assay and that was recently introduced into the clinic as a replacement for the original Trofile assay.

TABLE 4. Visit and time of first detection of CXCR4-using variants by MT-2 and Trofile assays

Patient identifier	Visit no. (time[a])			
	Original MT-2 assay[b] positive	Repeat MT-2 assay[c] positive	Original Trofile assay D/M	Enhanced-sensitivity Trofile assay D/M
13845	4		4 (0)	2 (−6)
13940	19	18 (−2)	21 (+7)	17 (−5)
13908	10		9 (−3)	9 (−3)
13912	12		12 (0)	12 (0)
13907	9		9 (0)	9 (0)
13885	11		11 (0)	11 (0)
13993	37	35 (−6)	38 (+3)	37 (0)
13904	22		25 (+3)	22 (0)
13951	22	21 (−3)	24 (+6)	22 (0)
13988	22	23 (+3)	26 (+11)	26 (+11)

[a] Time (in months) from time of detection by original MT-2 assay.
[b] Original MT-2 assay, results of the MT-2 assay performed with fresh patient PBMCs in real time.
[c] Repeat MT-2 assay, results of the MT-2 assay performed with cryopreserved patient PBMCs from the same time points in 2006.

These analyses demonstrate that the enhanced-sensitivity assay detected CXCR4 use at earlier time points than the original Trofile assay, more closely matching the results of the MT-2 assay. Therefore, it seems that the plasma virus population and the replication-competent virus population in PBMCs may actually be quite comparable with regard to the representation of CXCR4-using variants. As CXCR4-using variants first emerge, they apparently do not immediately dominate the virus population in either compartment (24), but rather, their presence may fluctuate for a time at levels near the detection limit of current assays. Moreover, when the luciferase signals were analyzed as a group, the luciferase signal for CXCR4-positive cells from the first patient samples classified as D/M by the original Trofile assay was significantly lower than the luciferase signal for subsequent D/M samples ($P = 0.0363$, unpaired t test). Thus, the sensitivity of the assay seemed to be a more important determinant of discordant findings than compartmentalization. To further illustrate this point, when samples were retested by the MT-2 assay, CXCR4-using variants were detected at time points 3 to 6 months different from the time of detection by the original MT-2 assay performed in real time. This also probably reflects the presence of CXCR4-using virus near the level of detection of the MT-2 assay, in agreement with the results of the enhanced-sensitivity Trofile assay. More than 6 months after their initial detection, the results for the detection of CXCR4-using

variants by all assays are mostly concordant, suggesting a continued higher-level presence of these variants.

It is conceivable that CXCR4-using variants are present at even earlier time points at levels below the detection limit of either assay. New sequencing technology with algorithm-based tropism predictions, such as massive parallel pyrosequencing, may allow us to investigate this further (10). However, the impact of CXCR4-using virus at extremely low levels remains to be established. The detection of CXCR4-using virus by both the MT-2 and the Trofile assays has been correlated with clinical outcomes, be it an accelerated $CD4^+$ T-cell decline in the absence of treatment (23) or the inferior virologic outcomes in individuals with CXCR4-using HIV commencing treatment with the CCR5 antagonist maraviroc (27). If CXCR4-using variants are indeed present for long periods of time at levels below the detection limit, it will be important to further investigate the parameters that determine whether or not they will achieve levels sufficient for detection and concomitant disease acceleration.

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