

Standardized Microtiter Assay for Determination of Syncytium-Inducing Phenotypes of Clinical Human Immunodeficiency Virus Type 1 Isolates

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A standardized assay in 96-well microtiter plates for syncytium-inducing (SI) human immunodeficiency virus type 1 phenotype detection using MT-2 cells has been developed. SI variants were found in 67% of the patients with advanced human immunodeficiency virus disease. The occurrence of the SI phenotype increased with lower CD4⁺ counts. There was no association between p24 antigenemia and the SI phenotype.

Syncytium-inducing (SI) human immunodeficiency virus type 1 (HIV-1) variants have been shown to be clinically significant in the pathogenesis of HIV-1 infection (1-3, 10, 12, 18). The ability of HIV-1 isolates to produce cytopathic effects in a human T-cell leukemia virus type I-transformed lymphoblastoid cell line (MT-2 cells) has been shown to be sensitive to and specific for SI capacity (5, 11). Within a single HIV-positive individual, a heterogeneous population of SI and non-SI (NSI) HIV-1 can be detected, and these virus populations shift over time (4, 13). A shift from a predominately monocytotropic NSI HIV-1 variant population to a T-cell tropic SI population has been associated with progression of HIV-1 disease (16). While SI viruses have been detected at all stages of HIV-1 infection, they are more commonly found among individuals with advanced disease (15, 17, 19). The presence of the SI phenotype is also correlated with accelerated CD4⁺ count declines (2, 10, 12, 14).

Many reported studies on the role of the SI phenotype in HIV-1 infection have examined syncytium formation induced by patients' peripheral blood mononuclear cells (PBMC) cocultivated with MT-2 cells in 25-cm² tissue culture flasks. This assay is cumbersome to perform on a large scale and is potentially subject to poor sensitivity because of the loss of viability over time of cryopreserved PBMC. We report the development and validation of a standardized 96-well microtiter assay for SI phenotype detection that is suitable for use with supernatants from HIV-1 cultures.

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Virus isolation and infectivity determination. PBMC from HIV-1-positive individuals were collected from anticoagulated

blood. HIV-1 was isolated according to standard techniques (6, 7). The National Institute of Allergy and Infectious Diseases AIDS Research and Reference Reagent Program distributed HIV-1 strains (A018A and A018C), kindly provided by Douglas Richman, and the human T-cell line MT-2 (derived from human leukemia virus type I-transformed T cells [5]). Virus stocks prepared from HIV-1 culture supernatants were stored as aliquots at -70°C. An infectivity assay was used to establish the 50% tissue culture infective dose (TCID₅₀) of the virus stock (8).

Standardized microtiter SI detection assay. Virus supernatant (50 µl or 200 TCID₅₀) was added to MT-2 cells (5 × 10⁴) in duplicate wells of flat-bottomed 96-well microtiter plates in a final volume of 200 µl per well. Plates were incubated at 37°C with 5% CO₂. Cultures were examined every 3 days for syncytium formation by using an inverted microscope until termination (14 to 28 days depending on the experiment). Syncytium formation was defined as three to five multinucleated giant cells per high-power field. The first day of positivity was documented. Following inspection, cultures were split by removing 130 µl of cells plus supernatant and replacing 150 µl of fresh medium in each well. If no syncytia were observed by the day of assay termination, the isolate was scored as NSI.

Flask method for SI detection. Virus supernatant (100 µl or 200 TCID₅₀) was added to 10⁶ MT-2 cells in a total volume of 5 ml in 25-cm² tissue culture flasks and incubated at 37°C with 5% CO₂. Syncytium formation was defined as three to five multinucleated giant cells per high-power field. Medium was exchanged on the same schedule as noted above for the microtiter plate assay.

The microtiter and tissue culture flask assays for syncytium formation in MT-2 cells were compared by assaying 53 isolates of HIV-1 in parallel. Fifty-one of 53 isolates (96.2%) were in complete agreement with respect to phenotype (39 SI and 12 NSI). Two isolates were NSI in the flask assay but SI in the microtiter assay. The median time to positivity was 6 days in the microtiter format compared with 8 days in the flasks (*P* = 0.0001, Wilcoxon signed-rank test).

The effect of a virus inoculum on the results in the microtiter

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TABLE 1. Effect of inoculum on SI phenotypic expression in 96-well microtiter assay

TCID ₅₀	n ^a	No. of days to appearance of syncytia		
		Median	Percentile	
			25th	75th
1	10	22	17	28 ^b
10	10	21	6	28
100	10	6	5	6
200	10	3	3	3
1,000	10	4	3	5
10,000 ^c	8	3	2	3

^a n, number of virus stocks assayed.

^b Three samples were negative after 28 days of observation.

^c Two virus stocks had insufficient titer to be assayed at this TCID₅₀.

assay was examined by infecting MT-2 cells with increasing TCIDs. For each isolate, the assay was performed in parallel at different inocula (1 to 10⁴ TCID₅₀ per 5 × 10⁴ MT-2 cells). There was an inverse relationship between the input inoculum and time to appearance of syncytia (*P* < 0.001, Page's test for ordered alternatives [Table 1]). These data suggested that when 50 μl of virus supernatant in the microtiter assay was used, the minimum allowable virus stock titer in a 14-day assay was 2,000 TCID₅₀ per ml. Previous studies have shown that ≥90% of virus stocks obtained by standard PBMC coculture methods yield a virus stock of ≥2,000 TCID₅₀ per ml (8).

To determine whether SI isolates have a growth advantage over NSI isolates during HIV-1 virus stock preparation and whether the use of these virus stocks would lead to misclassification because of expansion of small SI subspecies during the virus isolation, we compared SI and NSI results, using different

TABLE 2. Comparison of SI detection assay results

Patient no.	Result of SI detection assay ^a						
	A	B	C	D	E	F	G
1	+	NSI	NSI	+	NSI	ND	2
2	+	9	2	+	9	ND	2
3	+	9	2	+	11	ND	2
4	+	NSI	3	+	24	ND	NSI
5	+	27	2	+	11	ND	2
6	+	10	2	+	15	ND	NSI
7	+	10	2	+	19	ND	2
8	+	7	2	+	NSI	ND	NSI
9	+	15	7	+	NSI	ND	2
10	+	16	2	+	NSI	ND	3
11	+	12	2	+	NSI	ND	2
12	+	NSI	NSI	-	NSI	ND	NSI
13	+	NSI	NSI	+	NSI	NSI	ND
14	+	7	4	+	9	2	ND
15	+	NSI	NSI	+	NSI	NSI	ND
16	+	8	4	+	22	2	ND
17	+	NSI	NSI	+	NSI	NSI	ND
18	+	14	3	+	3	5	ND
19	+	6	4	+	4	2	ND
20	+	NSI	NSI	+	NSI	NSI	ND
21	+	NSI	NSI	+	NSI	NSI	ND
Median no. of days to positivity		10	2		11	2	2
% agreement with results in column B			95		76	100	75

^a Columns A to G refer to experimental results as depicted in Fig. 1. +, p24 antigen detected after cocultivation; numbers, day after culture on which SI cytopathic effect was first observed; ND, not done.

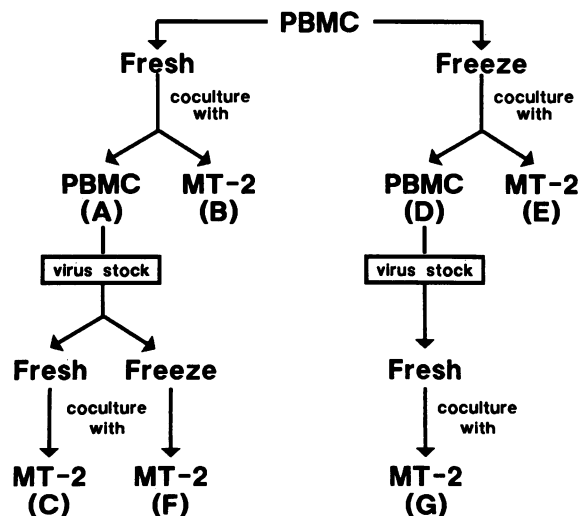


FIG. 1. Strategy to compare SI detection assay results with different specimens. Patients' PBMC (10⁶) were cocultured with either 2 × 10⁶ donor PBMC (A) or 5 × 10⁵ MT-2 cells (B). After 14 days, the virus stock from the PBMC coculture (A) was inoculated into MT-2 cells (C), or 10⁶ uncultured PBMC were frozen in liquid nitrogen for 7 to 10 days in 5 to 10% dimethyl sulfoxide. After 7 to 10 days in liquid nitrogen, PBMC were cocultivated with either PBMC (D) or MT-2 cells (E). Virus stock from fresh PBMC coculture (A) was frozen for 10 to 14 days and cocultivated with MT-2 cells (F). Virus stocks derived from cryopreserved PBMC that were thawed and cultured were cultivated with MT-2 cells (G).

patient-derived specimens. PBMC from 21 HIV-positive individuals were processed as depicted in Fig. 1. The data were analyzed by using the result obtained by cocultivation of fresh PBMC with MT-2 cells as the reference standard (Fig. 1, column B). This standard was chosen because it represents the viral phenotype before specimen handling (HIV-1 amplification and/or freezing) and was the assay originally described by Koot and coworkers (11). Results using virus stocks (Table 2, columns C and F) from fresh PBMC cocultures most closely agreed with the reference standard. The worst agreement was observed when PBMC were initially cryopreserved and then processed in culture (Table 2, columns E and G). Virus stock specimens, when SI, were positive earlier than PBMC specimens from the same patients (Table 2). The results indicated that the use of virus stocks in the microtiter assay format accurately reflected the presence of the SI phenotype in the patient.

Association of viral phenotype with CD4⁺ count and p24 antigenemia. Baseline virus isolates (*n* = 255) from subjects enrolled in the AIDS Clinical Trial Group 116B/117 were assayed for the SI phenotype (9). Enrollees in the AIDS Clinical Trial Group 116B/117 had a minimum of 16 weeks of previous zidovudine treatment and AIDS or AIDS-related complex with ≤300 CD4⁺ cells or asymptomatic HIV infection with ≤200 CD4⁺ cells at entry into the study. Virus was successfully recovered from 82% (208 of 255) of the PBMC cocultures. The median TCID₅₀ per milliliter for these virus supernatants was 2.6 × 10⁴ (6.5 × 10³ and 1.6 × 10⁵, 25th and 75th percentiles, respectively). A standard inoculum of 200

TABLE 3. Association of viral phenotype with CD4⁺ count and p24 antigenemia

Patient characteristic	% (no.) of individuals with indicated phenotype		Relative risk (95% CI) ^a
	SI	NSI	
CD4 ⁺ count ^b			
Baseline	67 (126)	33 (61)	
≤50	80 (59)	20 (15)	2.6 (1.4, 4.9)*
51–154	74 (42)	26 (15)	2.4 (1.3, 4.6)*
155–220	55 (18)	45 (15)	1.8 (0.9, 3.6)
≥221 ^c	30 (7)	70 (16)	1.0
p24 antigenemia			
Positive	67 (50)	33 (25)	0.96 (0.8, 1.2)
Negative ^c	69 (68)	31 (30)	1.0

^a The relative risk is the change in risk for SI phenotype in patients with a given factor (CD4⁺ count or p24 antigenemia status) compared to the reference gp. The relative risk is considered significant if the 95% confidence intervals do not include 1.0. Asterisks indicate significance.

^b CD4⁺ counts (number of cells per mm³) were categorized into quartiles on the basis of the distribution of CD4⁺ counts in the individuals with NSI results.

^c Reference group.

TCID₅₀ per 5 × 10⁴ MT-2 cells was used in the microtiter assay format. The median time to positivity for SI isolates was 3 days.

SI HIV-1 variants were found in 67% (126 of 187) of the individuals at baseline (Table 3). The median CD4⁺ count of individuals with SI variants was significantly lower than that of individuals with NSI variants (53/mm³ and 155/mm³, respectively [*P* < 0.001, Wilcoxon rank sum test]). A trend analysis showed that individuals with lower CD4⁺ counts were more likely to have SI variants (*P* < 0.001, Cochran-Armitage trend test). On the basis of the CD4⁺ count divided in quartiles among the individuals with NSI viruses, individuals in the groups with CD4⁺ counts of ≤50/mm³ and between 51/mm³ and 154/mm³ were significantly more likely to have SI variants than were individuals with CD4⁺ counts of ≥221/mm³ (relative risks, 2.6 and 2.4, respectively [Table 3]). Our results confirm previous findings that SI variants are more commonly detected in individuals with lower CD4⁺ counts. Nevertheless, 20% of individuals with CD4⁺ counts of ≤50/mm³ had NSI isolates. There was no association between the presence of SI isolates and p24 antigenemia (*P* = 0.74, Fisher's exact test [Table 3]).

In conclusion, the standardized microtiter assay for the detection of SI variants presented herein provides a uniform and inexpensive method for testing clinical HIV-1 isolates. A standardized assay is essential for use in multicenter studies. While it does not appear necessary to determine the titers of all HIV-1 virus stocks for use in the assay, false negative findings (interpreted as NSI) can result from the use of a noninfectious virus supernatant. In cases in which the infectivity of the virus stock is not known, a control infection of PBMC is appropriate to confirm an NSI interpretation.

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