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

ANTHONY J. JAPOUR,[1]* SUSAN A. FISCUS,[2] JEAN-MARIE ARDUINO,[3] DOUGLAS L. MAYERS,[4] PATRICIA S. REICHELDERFER,[5] AND DANIEL R. KURITZKES[6]†

Division of Infectious Diseases, Department of Medicine, Beth Israel Hospital, and Harvard Medical School,[1] and Statistical Data Analysis Center, Harvard School of Public Health,[3] Boston, Massachusetts 02215; Department of Microbiology & Immunology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599[2]; Department of HIV Drugs and Gene Therapy, Walter Reed Army Institute of Research and Naval Medical Research Institute, Rockville, Maryland 20850[4]; Division of AIDS, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20892[5]; and Division of Infectious Diseases, University of Colorado Health Sciences Center and Veterans Affairs Medical Center, Denver, Colorado 80262[6]

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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 heterogenous 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-cm^2 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 Infections 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 ($TCID_{50}$) of the virus stock (8).

Standardized microtiter SI detection assay. Virus supernatant (50 μl or 200 $TCID_{50}$) was added to MT-2 cells (5×10^4) 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% CO_2. 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 $TCID_{50}$) was added to 10^6 MT-2 cells in a total volume of 5 ml in 25-cm^2 tissue culture flasks and incubated at 37°C with 5% CO_2. 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

* Corresponding author. Mailing address: Division of Infectious Diseases, Beth Israel Hospital, Dana 617, 330 Brookline Ave., Boston, MA 02215. Phone: (617) 735-5540. Fax: (617) 735-5541. Electronic mail address: tjapour@bih.harvard.edu.

† All authors are members of the AIDS Clinical Trials Group Virology Committee MT-2 Group, which also includes Daniella Livnat, Victoria Johnson, and Phil LaRussa.

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 $TCID_{50}$.

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 10^4 $TCID_{50}$ per 5×10^4 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 $TCID_{50}$ per ml. Previous studies have shown that ≥90% of virus stocks obtained by standard PBMC coculture methods yield a virus stock of ≥2,000 $TCID_{50}$ 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

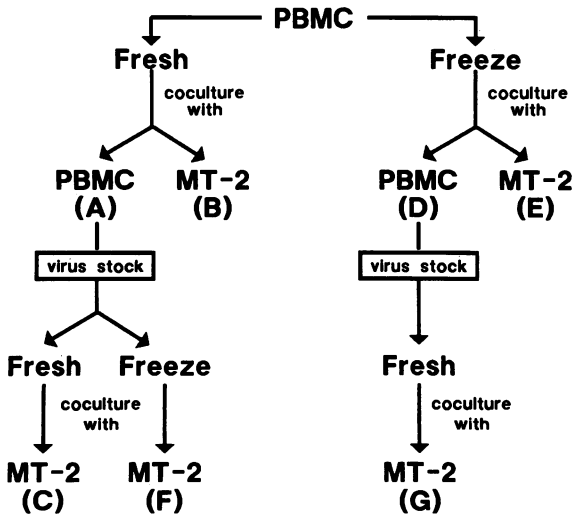

FIG. 1. Strategy to compare SI detection assay results with different specimens. Patients' PBMC (10^6) were cocultured with either 2×10^6 donor PBMC (A) or 5×10^5 MT-2 cells (B). After 14 days, the virus stock from the PBMC coculture (A) was inoculated into MT-2 cells (C), or 10^6 uncultured PBMC were 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).

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.

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 $TCID_{50}$ per milliliter for these virus supernatants was 2.6×10^4 (6.5×10^3 and 1.6×10^5, 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 mm^3) were categorized into quartiles on the basis of the distribution of $CD4^+$ counts in the individuals with NSI results.

[c] Reference group.

$TCID_{50}$ per 5×10^4 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 ($53/mm^3$ and $155/mm^3$, 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 $\leq 50/mm^3$ and between $51/mm^3$ and $154/mm^3$ were significantly more likely to have SI variants than were individuals with $CD4^+$ counts of $\geq 221/mm^3$ (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 $\leq 50/mm^3$ 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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REFERENCES

1. **Boucher, C. A. B., J. M. A. Lange, F. Miedema, G. J. Weverling, M. Koot, J. W. Mulder, J. Goudsmit, P. Kellam, B. A. Larder, and M. Tersmette.** 1992. HIV-1 biological phenotype and the development of zidovudine resistance in relation to disease progression in asymptomatic individuals during treatment. AIDS **6:**1259–1264.
2. **Bozzette, S. A., A. McCutchan, S. A. Spector, B. Wright, and D. R. Richman.** 1993. A cross-sectional comparison of persons with syncytium- and non-syncytium-inducing human immunodeficiency virus. J. Infect. Dis. **168:**1374–1380.
3. **D'Aquila, R. T., V. Johnson, S. Welles, A. Japour, D. Kuritzkes, V. DeGruttola, P. Reichelderfer, R. Coombs, C. Crumpacker, J. Kahn, and D. Richman.** Zidovudine resistance and human immunodeficiency virus type 1 disease progression during antiretroviral therapy. Submitted for publication.
4. **Groenink, M., R. A. M. Fouchier, R. E. Y. de Goede, F. de Wolf, R. A. Gruters, T. M. Cuypers, H. G. Huisman, and M. Tersmette.** 1991. Phenotypic heterogeneity in a panel of infectious molecular human immunodeficiency virus type 1 clones derived from a single individual. J. Virol. **64:**1968–1975.
5. **Harada, S., Y. Koyanagi, and N. Yamamoto.** 1985. Infection of HTLV-III/LAV in HTLV-I-carrying cells MT-2 and MT-4 and application in a plaque assay. Science **229:**563–566.
6. **Hollinger, F. B., J. W. Bremer, L. E. Myers, J. W. M. Gold, L. McQuay, and The NIH/NIAID/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.
7. **Jackson, J. B., R. W. Coombs, K. Sannerud, F. S. Rhame, and H. H. Balfour, Jr.** 1988. Rapid and sensitive viral culture method for human immunodeficiency virus type 1. J. Clin. Microbiol. **26:**1416–1418.
8. **Japour, A. J., D. L. Mayers, V. A. Johnson, D. R. Kuritzkes, L. A. Beckett, J. M. Arduino, J. Lane, R. J. Black, P. S. Reichelderfer, R. T. D'Aquila, C. S. Crumpacker, and The AIDS Clinical Trials Group Virology Committee Resistance Working Group.** 1993. Standardized peripheral blood mononuclear cell culture assay for determination of drug susceptibilities of clinical human immunodeficiency virus type 1 isolates. Antimicrob. Agents Chemother. **37:**1095–1101.
9. **Kahn, J. O., S. W. Lagakos, D. D. Richman, A. Cross, C. Pettinelli, S. Liou, M. Brown, P. A. Volberding, C. S. Crumpacker, G. Beall, H. S. Sacks, T. C. Merrigan, M. Beltangady, L. Smaldone, R. Dolin, and The NIAID AIDS Clinical Trials Group.** 1992. A controlled trial comparing continued zidovudine with didanosine in human immunodeficiency virus infection. N. Engl. J. Med. **327:**581–587.
10. **Koot, M., I. P. M. Keet, A. H. V. Vos, R. E. Y. de Goede, M. T. L. Roos, R. A. Coutinho, F. Miedema, P. T. A. Schellekens, and M. Tersmette.** 1993. Prognostic value of HIV-1 syncytium-inducing phenotype for rate of CD4+ cell depletion and progression to AIDS. Ann. Intern. Med. **118:**681–688.
11. **Koot, M., A. H. V. Vos, R. P. M. Keet, R. E. de Goude, 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.
12. **Richman, D. D., and S. A. Bozzette.** The impact of the syncytium-inducing phenotype of human immunodeficiency virus on disease progression, in press.
13. **Sakai, K., S. Dewhurst, X. Ma, and D. J. Volsky.** 1988. Differences in cytopathogenicity and host cell range among infectious molecular clones of human immunodeficiency virus type 1 simultaneously from an individual. J. Virol. **62:**4078–4085.
14. **Schellekens, P. T. A., M. Tersmette, M. T. L. Roos, R. P. Keet, F. de Wolf, R. A. Coutinho, and F. Mediema.** 1992. Biphasic rate of CD4+ cell count decline during progression to AIDS correlates with HIV-1 phenotype. AIDS **6:**665–669.

15. **Schuitemaker, H., M. Koot, N. A. Kootstra, R. Y. de Goede, F. de Wolf, F. Miedema, and M. Tersmette.** 1991. Monocytotrophic human immunodeficiency virus type 1 (HIV-1) variants detectable in all stages of HIV-1 infection lack T-cell line tropism and syncytium-inducing ability in primary T-cell culture. J. Virol. **65:** 356–363.
16. **Schuitemaker, 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. Eeftink Schattenkerk, 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 populations. J. Virol. **66:**1354–1360.
17. **Schuitemaker, H., N. Kootstra, M. Groenink, R. E. Y. de Goede, F. Miedema, and M. Tersmette.** 1992. Differential tropism of clinical HIV-1 isolates for primary monocytes and promonocytic cell lines. AIDS Res. Hum. Retroviruses **8:**1679–1682.
18. **St. Clair, M. H., P. M. Hartigan, J. C. Andrews, C. L. Vavro, M. S. Simberkoff, and J. D. Hamilton.** 1993. Zidovudine resistance, syncytium-inducing phenotype, and HIV disease progression in a case-control study. J. Acquired Immune Defic. Syndr. **6:**891–897.
19. **Tersmette, M., R. E. Y. de Goede, B. J. M. Al, I. N. Winkel, R. A. Gruters, H. T. Cuypers, H. G. Huisman, and F. Miedema.** 1988. Differential syncytium-inducing capacity of human immunodeficiency virus isolates: frequent detection of syncytium-inducing isolates in patients with acquired immunodeficiency syndrome (AIDS) and AIDS-related complex. J. Virol. **62:**2026–2032.