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# Evaluation of an Infectivity Standard for Real-Time Quality Control of Human Immunodeficiency Virus Type 1 Quantitative Microculture Assays

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**Quantitative microculture assays of cryopreserved human immunodeficiency virus type 1-infected cell suspensions and culture supernatants were compared among seven assay sites. There was no significant change in titer during 1 year of storage. The overall standard deviation for infected cell suspensions was approximately 0.8 $\log_{10}$ virus titer. A method for detecting deviant assay results was developed and was used to identify two donor cell preparations ($n = 54$) that gave consistently low titers.**

Human immunodeficiency virus type 1 (HIV-1) can be cultured from peripheral blood mononuclear cells (PBMCs) from virtually all HIV-1-infected patients (1–3, 15–18), and infectious viral load can be quantitated by coculturing successive dilutions of patient cells (3, 5, 8, 12, 15, 28) or plasma (3, 4, 15, 19, 22, 27) with uninfected donor PBMCs. The precision of these measurements is limited by differences in susceptibility to HIV infection between preparations of donor PBMCs (9, 11, 29–31) and other technical factors.

The purpose of the study described here was to evaluate the inter- and intralaboratory reproducibilities of the AIDS Clinical Trials Group (ACTG)-standardized quantitative microculture assay of centrally produced supplies of HIV-1-infected cells and cell-free virus and assess the stabilities of these reagents during storage.

**Infected cells and cell-free virus.** A total of $5.4 \times 10^8$ PBMCs were stimulated with phytohemagglutinin (PHA; 5 μg/ml; Sigma) for 48 h, infected with 4,800 50% tissue culture infective doses of HIV-$1_{JR-CSF}$ stock (virus strain from Irwin Chen through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases [20, 26]), and grown in six flasks in coculture medium (RPMI 1640 supplemented with glutamine [0.3%], interleukin-2 [5%], penicillin [100 U/ml], streptomycin [100 μg/ml], and heat-inactivated fetal bovine serum [20%]) (6). Culture supernatants were pooled on day 10, centrifuged twice, and stored in 1-ml aliquots at −70°C (media-UL) or in the vapor phase of liquid nitrogen (media-LN). The cell pellet was resuspended, washed twice in phosphate-buffered saline, cryopreserved in aliquots containing $3 \times 10^6$ cells in 1 ml of coculture medium supplemented with 10% dimethyl sulfoxide, and stored in the vapor phase of liquid nitrogen (cells-LN) or at −70°C (cells-UL). Viability at the time of harvest was greater than 90%. Forty-eight days after the specimens were prepared, they were shipped on dry ice to the participating laboratories, where they were stored at the same temperature at which they were stored prior to shipment.

**Titrations.** Within 12 days after receipt of the specimens and at 4-month intervals for 1 year, each assay site performed titrations in duplicate: one assay for each specimen type and storage condition with cells from one donor and a second assay with cells from a second donor. Donor PBMCs were isolated from HIV-1-seronegative blood obtained from local blood banks (for six laboratories) or from donors preselected to support HIV-1 culture (for one laboratory) and stimulated with PHA (5 μg/ml; Difco or Sigma) for 1 to 3 days prior to use for coculture (6, 16).

Aliquots of the infected cell suspensions were thawed and resuspended in coculture medium (with penicillin-streptomycin or with gentamicin [50 μg/ml]). Percent viability was determined by trypan blue exclusion, and HIV titers, in infectious units per million viable cells (IUPM), were determined by the

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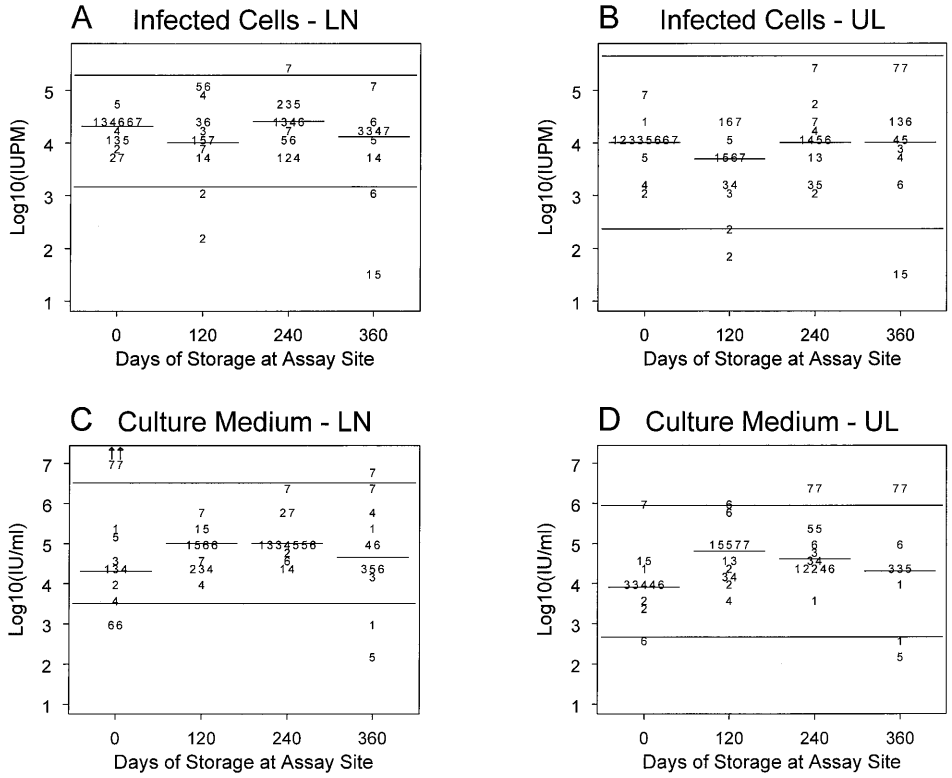


FIG. 1. Reproducibility of estimates of $\log_{10}$ virus titer. Data are provided for duplicate titration assays performed at seven assay sites (designated 1 to 7) with HIV-$1_{JR\text{-}CSF}$-infected PBMCs (A and B) or culture supernatants (C and D) at various times after storage in liquid nitrogen (A and C) or at −70 to −90°C (B and D). Time zero represents the time of the first titration at the assay site (between 50 and 60 days after the specimens were prepared in the central laboratory). Arrows (C) indicate data obtained from dilution series that did not reach the endpoint. The medians for all assays at each time point are indicated by short horizontal lines. Long horizontal lines in each panel represent the upper (median titer + $2S_r$) and lower (median titer − $2S_r$) limits used to detect deviant assay results with respect to the overall distribution. $S_r$, the robust estimate of SD (see text), was determined separately for each combination of specimen type and storage condition.

standard ACTG quantitative micrococulture procedure (6). IUPM was calculated from the pattern of positive cultures in each assay by the method of maximum likelihood (25). Aliquots of culture supernatants were thawed and diluted in coculture medium. HIV titers, in infectious units (IU) per milliliter of the original culture supernatant, were determined as described for the infected cell suspensions. The *P* value for goodness-of-fit (PGOF) of the observed distribution of positive wells, given the estimated titer, was also calculated for each assay (25). Eleven assays ($n = 215$) were excluded from the analyses because PGOF values of <0.05 signified that the results were very unlikely according to the model (25), indicating that these assay results were unreliable.

**Dependence of HIV-1 titer and infected cell viability on storage conditions and storage time at the assay sites.** The results for all titrations are presented in Figure 1. From 9 to 14 determinations with PGOFs of ≥0.05 were obtained at each time point for each combination of specimen type and storage condition. Two titrations did not reach the endpoint, and minimum viral titers were estimated by assuming that if the assay had been extended by one dilution, an endpoint would have been reached (10).

The median titer for infected cell specimens ranged from 5,000 to 26,000 IUPM and appeared to be independent of storage condition (Fig. 1A and B). The median titer for culture medium specimens ranged from 8,000 to 100,000 IU/ml and also did not differ between storage conditions (Fig. 1C and D). No significant change in titer was observed over the period studied for any of the specimens (random effects model [21] for cells-LN, $\Delta\log_{10}$ IUPM = −0.13 per 120 day period; 95% confidence interval, −0.33 to +0.07; the loss of titer for the other specimens was less than 0.13). During the 360-day period of the study, the median viability of cells stored in the vapor phase of liquid nitrogen dropped from 83.3 to 69.7%, and for cells stored at −70 to −90°C, median viability dropped from 77.3 to 64.3% (Fig. 2), which represents a significant downward trend for the combined data ($P = 0.014$; repeated measures

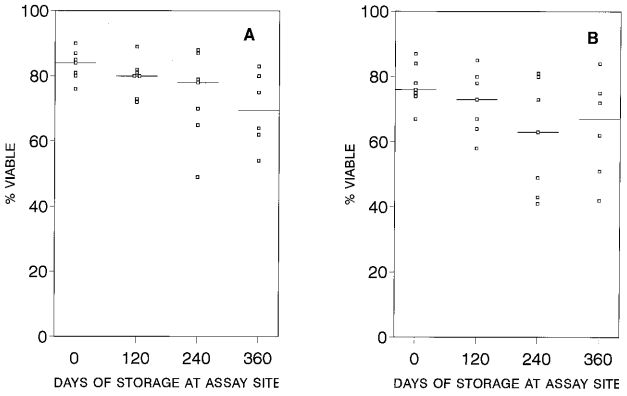


FIG. 2. Changes in viability of HIV-$1_{JR\text{-}CSF}$-infected PBMC suspensions during storage in liquid nitrogen (A) or at −70 to −90°C (B). Percent viability was determined by trypan blue exclusion by each laboratory prior to carrying out the titrations shown in Fig. 1.

TABLE 1. Comparison of intralaboratory and total variabilities in titration results[a]

| Days of storage at assay site | Comparison | SD of $\log_{10}$ virus titer | | | |
|---|---|---|---|---|---|
| | | Infected cell | | Culture medium | |
| | | LN | UL | LN | UL |
| 0 | Intralaboratory | 0.32 | 0.42 | 0.40[b] | 0.46 |
| | Total | 0.30[c] | 0.44 | 2.20[b] | 0.56 |
| 120 | Intralaboratory | 0.55 | 0.39 | 0.41 | 0.26 |
| | Total | 0.81 | 0.80 | 0.53 | 0.67 |
| 240 | Intralaboratory | 0.56 | 0.64 | 0.41 | 0.28 |
| | Total | 0.50[c] | 0.69 | 0.57 | 0.82 |
| 360 | Intralaboratory | 1.05 | 1.12 | 0.97 | 0.89 |
| | Total | 1.09 | 1.24 | 1.32 | 1.56 |
| All time points | Intralaboratory | 0.66 | 0.71 | 0.61[b] | 0.48 |
| | Total | 0.74 | 0.84 | 1.25[b] | 0.96 |

[a] Calculated from a restricted database of 194 assays. To allow calculation of intralaboratory and total variabilities from the same database, results from an assay site were excluded for any time point for which assays were carried out with less than two donor cell preparations.

[b] Two assays that did not reach the endpoint (Fig. 1C) were assigned equal values, as described in the text. Since the actual titers are not known and may not be equal to each other, both total and intralaboratory SDs are minimum estimates.

[c] A total SD that is smaller than the intralaboratory SD presumably reflects imprecision in the estimation procedure evident only when the difference between total and intralaboratory SDs is small.

method [24]), with no significant difference in the change between the storage conditions ($P = 0.72$).

Our results imply that infectivity standards could be prepared, shipped, and stored for at least a year without a loss of titer. These findings also may have implications for the reliability of assays performed with clinical specimens that have been stored frozen; however, the applicability of these results for HIV-$1_{JR\text{-}CSF}$ to clinical specimens remains to be tested.

**Reproducibility of the titration assay.** The total and intralaboratory variabilities of the assay results are provided for each time point and for all time points combined (Table 1). For infected cell suspensions, the total standard deviation (SD) for all time points combined was only slightly greater than the intralaboratory SD. ACTG laboratories have followed a standardized procedure for determining the titers of infected cell suspensions since 1992 (6, 13, 16), and the finding that agreement among laboratories was almost as good as agreement between assays carried out in the same laboratory with different donor cells attests to the effectiveness of these standardization procedures.

Compared with titrations performed with infected cells, total variability was greater for assays performed with culture media, but intralaboratory variability was not increased. This suggests that the assay was not intrinsically more variable when it was performed with cell-free virus but, rather, that it was less well standardized between laboratories than the assay performed with infected cell suspensions. Variability may be influenced by the procedures used for thawing or diluting virus stocks and differences in culture conditions which affect the stability of cell-free virus particles. Similar interlaboratory variability for HIV-1 stock titrations was recently reported (7) in a comparison among nine laboratories.

**Detection of deviant assay results.** Estimates of viral titer that differed from the median by more than 2 SDs were classified as deviant. Robust estimates of SD ($S_r$) were used for this classification [$S_r = (Q_3 - Q_1)/1.33$, where $Q_1$ and $Q_3$ are the 25th and 75th percentiles of the viral titer, respectively]. Since viral titer did not change significantly during storage, the results were pooled across time points to estimate $Q_1$, $Q_3$, and the median for each combination of specimen type and storage condition. Estimates of the median $\pm 2S_r$ are plotted in Fig. 1.

Ten values greater than $\pm 2S_r$ would be expected from a normal distribution of the 204 titration results; however, 26 were observed (Fig. 1). Twelve of the 26 values were from assays performed on day 360 and 8 were derived from two preparations of donor PBMCs. These two donor PBMC preparations consistently gave the lowest titers for each combination of specimen type and storage condition, while duplicate titrations carried out by the same laboratories with different donor PBMC preparations gave values near the median. Other deviant assay results tended to occur more frequently in culture medium titrations than in infected cell titrations. The distribution among the four intervals of storage was approximately even.

Our finding that only 2 of 54 donor cell preparations gave consistently deviant assay results suggests that HIV-$1_{JR\text{-}CSF}$ is satisfactory for use as an infectivity standard; however, it might not identify donor cell preparations uniquely restricted in their ability to replicate a subset of clinical isolates, and donor cells which replicate HIV-$1_{JR\text{-}CSF}$ poorly may still support good growth of some clinical isolates. An 18-fold range in HIV p24 antigen production among nine different donor cell preparations infected with HIV-$1_{JR\text{-}CSF}$ was reported in a recent study (29). Low-passage clinical isolates and biological clones tested with the same donor cell panel in that study showed less variability.

Variability in titer of infected PBMCs in blood drawn from the same patient a few days apart ranged from 0.63 to 0.80 $\log_{10}$ IUPM for three large clinical trials including ACTG protocol 143 (10) and protocols 175 and 241 (16a). A major component of this within-subject variability may be accounted for by intralaboratory variability in the assay (0.66 to 0.71 $\log_{10}$ IUPM in the present study). Parallel assays with a reference infectivity standard should allow us to identify deviant assay results and improve the precision of the assay. To test this, the variability for infected cell suspensions was recalculated after removing the results for assays with the two donor cell preparations that consistently gave deviant results. The resulting intralaboratory SD of the $\log_{10}$ IUPM over all time points for cells-LN was 0.51, and for cells-UL it was 0.49, suggesting that significant improvement is possible through implementation of real-time quality control in this assay. The total SD of the $\log_{10}$ IUPM was also reduced (for cells-LN, 0.57; for cells-UL, 0.72; mean, 0.65). For a two-arm clinical trial in which treatment effect is evaluated by comparing one pre- and one posttreatment measurement, a reduction in the SD of the $\log_{10}$ IUPM from 0.8 to 0.65 would result in a proportional decrease in the magnitude of the effect that could be detected. Alternatively, this improvement in assay precision would allow a 34% reduction in sample size with no loss of power to detect a treatment effect.

**Suggested procedure for real-time quality control.** The lower variability that we observed with infected-cell suspensions leads us to recommend the use of cryopreserved HIV-$1_{JR\text{-}CSF}$-infected cells as an infectivity standard. Aliquots of the infectivity reference standard with known titers should be thawed and assayed in parallel with clinical specimens. Minimally, this should be done for each new donor cell preparation. Four culture wells containing two high-titer standards and two low-titer standards should be set up in parallel with the clinical

specimens to be assayed. All results for clinical specimens obtained with donor cells for which both high-titer standard wells are negative or both low-titer standard wells are positive should be rejected as unreliable. On the basis of the results of the present study, a high-titer standard of 25 IU and a low-titer standard of 0.04 IU would reject two assays for cells-LN and four assays for cells-UL (including the four assays performed with the donor cell preparations which consistently performed poorly). More stringent quality control would be achieved by using 15 IU in the high-titer standards and 1/15 IU in the low-titer standards at a cost of rejecting more assays.

Quantitative cell culture measures functional virus, which may or may not correlate with the levels of HIV-1 RNA in plasma. Recent data from natural history studies (14) have demonstrated that culturable virus increases with disease progression, while plasma RNA levels, although prognostic, appear to remain constant (23). This suggests that multiple virologic and immunologic measurement parameters will be necessary to accurately assess HIV-1 disease pathogenesis.

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