Optimization of Quantitative Culture Assay for Human Immunodeficiency Virus from Plasma

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Experimental conditions essential to a simple, reproducible, quantitative human immunodeficiency virus plasma assay were determined. Five parameters were evaluated: length of culture, reproducibility, assay dilution schema, washing procedures, and anticoagulant usage. The recommended quantitative plasma assay utilizes undiluted citrated plasma cultured with peripheral blood mononuclear cells for 14 days with fivefold dilutions and a medium change on day 1 with no washing.

Quantification of human immunodeficiency virus type 1 (HIV-1) is a useful virologic marker for assessing the level of disease and possibly the efficacy of antiretroviral therapy. HIV-1 can be isolated from peripheral blood mononuclear cells (PBMC) of almost all HIV-1-infected patients (1,5). The virus isolated from PBMC, however, may not necessarily represent active virus replication in vivo, because most culture techniques are devised to reengineer latent cellular virus. Conversely, plasma viremia represents the release of in vivo-replicating HIV-1; thus, virus isolated from plasma represents active replication in vivo. This aspect should be particularly useful when antiretroviral agents like protease inhibitors are evaluated for therapy, because their function is to inhibit cleavage of viral structural proteins and not replication, virus can still be produced but it will be rendered noninfectious (7). Thus, PBMC viral cultures would remain positive while plasma cultures become negative during effective therapy. Furthermore, plasma viremia has been associated with disease progression and response to other antiretroviral therapies (1,5,9,10), making it a desirable assay for monitoring therapy. While several culture methods have been developed (1,3,5,9), there is no standard protocol for quantification of infectious HIV-1 from plasma. In addition, there is no consensus for the use of one, standard anticoagulant for blood collection. Both heparin and citrate have been used with good to limited success (1,3,9). With the development of RNA PCR techniques for quantification of viral RNA in plasma, it would be an advantage to use a universal anticoagulant which would allow both plasma culture and RNA PCR to be performed on the same sample. Heparin, but not citrate, has been shown to inhibit PCR (6). The purpose of this study was to develop a sensitive consensus protocol for quantification of HIV-1 present in plasma, by use of one universal anticoagulant, which could be performed reproducibly in clinical trials across laboratories.

In order to perform these assays, blood was collected from HIV-1-seropositive volunteers with CD4+ cell counts of \( \geq 400/\mu l \) recruited at random by each of the participating AIDS Clinical Trials Group plasma viremia laboratories. Blood was collected in tubes containing one of the anticoagulants heparin or acid citrate dextrose (ACD). Plasma was separated within 6 h after venipuncture by centrifugation at 400 to 800 \( \times \) 20 min. The separated plasma was removed and recentrifuged at 800 \( \times \) g for an additional 20 min to remove platelets and cell debris. The plasma was either used immediately for culture or frozen at \( -70^\circ C \) and cultured at a later time (2,11). Each laboratory used only samples collected at its site.

The AIDS Clinical Trials Group quantitative plasma viremia protocol under evaluation recommended that cultures for HIV-1 be set up in duplicate in 24-well tissue culture plates with 6 fivefold dilutions of plasma. Each initial sample or dilution (400 \( \mu l \)) was cultured with 2 \( \times \) 10\(^6\) 1- to 3-day-old phytolactaglucitin (PHA)-stimulated HIV-1-seronegative PBMC (1) in 1.6 ml of growth medium (RPMI with 20% fetal calf serum, 5% interleukin-2, and antibiotics [either 1% penicillin with 1% streptomycin or 50 \( \mu g \) of gentamicin per ml]). At 7 days, fresh growth medium containing 0.5 \( \times \) 10\(^6\) PHA-stimulated PBMC was added to the cultures. Supernatant from each well was harvested at 14 days and assayed for HIV-1 p24 antigen by a standard p24 enzyme-linked immunosorbent assay (ELISA) (Abbott Laboratories, North Chicago, Ill., or Coulter Immunology, Hialeah, Fla.). Any well with more than 30 pg of p24 antigen per ml was considered positive (4). Titers were determined as the reciprocal of the highest dilution of plasma yielding a positive culture. All laboratories participating in experiments to optimize the quantitative HIV-1 plasma assay

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were certified to perform HIV-1 quantitative cultures and HIV-1 p24 antigen ELISA in the AIDS Clinical Trials Group virology quality assurance program. Variations in culture time, dilution schema, and washing procedures were evaluated in order to optimize the assay conditions. Differences in endpoint titers among assays were analyzed by nonparametric measures for matched samples with the Friedman test and the Wilcoxon signed rank test by use of the Instat Program, GraphPad Software, Inc. (San Diego, Calif.). Differences between the number of samples per endpoint titer in any one test group and those in the others were determined by contingency table analysis with the chi-square test. Significant correlations among the data were determined with linear regression or multiple linear regression with the Microstat Program, Eco-soft, Inc. (Indianapolis, Ind.).

In order to evaluate whether 14 days of culture were sufficient for determination of the infectious titer of HIV-1 in plasma, the duration of plasma microcultures was extended an additional 14 days with $0.5 \times 10^6$ PHA-stimulated PBMC and fresh growth medium was added every 7 days. Supernatants were assayed for p24 antigen, and titers were determined on days 14, 21, and 28 postinfection. There was no significant difference in the numbers of positive cultures confirmed by prolonged incubation: 13, 15, or 14 out of 27, respectively ($P = 0.86$, chi-square test). In positive samples, there was no significant increase in endpoint titers: mean ± standard deviation = $118 \pm 78$ on day 14, $131 \pm 78$ on day 21, and $131 \pm 78$ on day 28 ($P = 0.63$, Friedman test). Therefore, there was no advantage in retaining and assaying of cultures beyond 14 days.

To assess the variability that existed because of differences in technical manipulation and PBMC sensitivity among donors, a single plasma sample was collected, aliquoted, frozen at $-70^\circ$C, and assayed 22 times at a single site with PBMC from multiple donors over a 36-week period. The median titer was 625, with a range between 125 and 3,125. Thus, the titer obtained from the quantitative plasma assay was reproducible within a single fivefold dilution (0.7 log units).

Although addition of 400 $\mu$L of undiluted plasma to the initial wells of the assay plate would increase sensitivity, undiluted plasma may contain inhibitory components which could interfere with the culture of HIV-1 in vitro (8). To investigate this further, plasma samples collected from 21 individuals were assayed. Triplicate samples were set up (i) with the initial plasma diluted 1:5, (ii) with the initial plasma undiluted with a PBMC wash on day 1, or (iii) with the initial plasma undiluted without a wash step and only a medium change on day 1. The wash step consisted of centrifugation of PBMC with three medium changes. There were no significant differences in the endpoint titers ($P = 0.61$, Friedman test) or the ranges of titers ($P = 0.70$, chi-square test) among the three assays evaluated, and the values were correlated ($P < 0.001$, multiple linear regression) (Fig. 1 and 2). There was 90% agreement (19 of 21) within 1 fivefold dilution (0.7 log unit) between the method with undiluted plasma and that with a 1:5 dilution of plasma in the initial wells. The agreement between assays when undiluted plasma was used with and without PBMC washing was 85% (18 of 21) ($P = 0.87$, chi-square test). Thus, neat plasma could be used to increase the sensitivity of
the assay without inhibitory effects and without an increased requirement for washing.

To ensure that the method used for washing PBMC did not affect the endpoint titters, 21 independent cultures, set up in duplicate, were washed on day 1 by centrifugation either in tubes or in 24-well culture plates. There were no significant differences in endpoint titters \((P = 0.56, \text{ Wilcoxon test})\) or the ranges of titters \((P = 0.75, \text{ chi-square test})\), and the titters were correlated \((P = 0.007, \text{ linear regression})\) (Fig. 1 and 2). There was 85% \((18 \text{ of } 21)\) agreement between titters within 1 dilution attained with either wash procedure. Thus, plate washing and tube washing yielded the same results.

Because coagulation may occur in wells with a higher concentration of plasma, cell loss during the medium change was a concern. Thus, we determined whether the addition of PBMC at this time would improve culture conditions and result in increased titters. Twenty plasma cultures were set up in duplicate. Both replicates were cultured starting with 400 μl of undiluted plasma. In one of the replicate cultures, 0.5 × 10⁶ PBMC were added at the time of the day 1 medium change. The other received only growth medium. There were no significant differences in the endpoint titters \((P = 0.73, \text{ Wilcoxon test})\) or the ranges of titters \((P = 0.75, \text{ chi-square test})\), and the titters were correlated \((P = 0.003, \text{ linear regression})\) (Fig. 1 and 2). Therefore, the addition of PBMC on day 1 did not improve the HIV-1 plasma viremia titer.

Blood collected for viral culture has usually been anticoagulated with heparin. However, assays for HIV-1 nucleic acids are usually performed on plasma anticoagulated with citrate because heparin can inhibit the PCR \((6)\). Ideally, PCR and culture should be performed from the same sample. This is particularly important for pediatric specimens, because blood volume is limited and obtaining more than one sample is not practical. Therefore, to determine if the anticoagulant influenced infectious HIV-1 recovery from plasma, 24 blood samples were collected in both heparin- and ACD-containing Vacutainer tubes. Assays were set up simultaneously with 400 μl of undiluted plasma in the initial wells and without a wash step. There was not a significant difference in the range of titers between plasma collected in heparin and plasma collected in ACD \((P = 0.11, \text{ chi-square test})\), and the titters were correlated \((0.01, \text{ linear regression})\) (Fig. 1 and 2). However, when HIV-1 titers in heparinized plasma were ≤25, with a mean of 7, there were significantly higher titters observed in citrated plasma, with a mean of 591 \((n = 11, P = 0.01, \text{ Wilcoxon test or Mann-Whitney test})\). There were no significant differences between samples with titters of >25 \((n = 13, P = 0.46, \text{ Wilcoxon test})\). Because ACD HIV-1 titters tended to be higher, there was only a 75% \((18 \text{ of } 24)\) agreement between the titers measured by use of the two anticoagulants. Thus, HIV-1 can be cultured from heparinized or citrated plasma.

Titters, however, may be higher in plasma with lower concentrations of HIV-1 when ACD is used as the anticoagulant.

The increased titers observed with the anticoagulant ACD in this system differ from results previously reported by Dewar et al. \((3)\). They observed only 4 of 7 positive cultures when blood
was anticoagulated with citrate, compared with 6 of 7 positive cultures when heparin was used. There are several differences in their procedure which may account for the apparent discrepancies. (i) Sodium citrate and not ACD was used as an anticoagulant. (ii) A 1-ml sample of undiluted plasma, compared with 400 μl of plasma plus 1.6 ml of medium, was added directly to PBMC cultures. (iii) The plasma was used within 20 min, compared with 6 h or after freezing. (iv) The sample size was smaller, 7 compared with 24.

On the basis of our investigation, the following consensus protocol is recommended. Quantitative plasma viremia cultures for HIV-1 should be set up in duplicate in 24-well tissue culture plates with 6 fivefold dilutions of plasma from blood anticoagulated with ACD. Each initial sample or dilution (400 μl) is cultured with $2 \times 10^6$ PHA-stimulated 1- to 3-day-old HIV-1-seronegative PBMC (1) in 1.6 ml of growth medium (RPMI with 20% fetal calf serum, 5% interleukin-2, and antibiotics). Half the medium is removed on day 1, and fresh growth medium is added. Cultures are fed at 7 days, and fresh growth medium is added with $0.5 \times 10^6$ PHA-stimulated PBMC. The supernatant from each well is harvested at 14 days postinfection and assayed for HIV-1 p24 antigen by a standard p24 ELISA. Changes in titer of 2 fivefold dilutions or 1.4 log unit or more are considered significantly different.

In conclusion, we have described a simple culture assay for the quantitation of HIV-1 in plasma. The use of ACD as the anticoagulant increases the utility of the plasma sample, allowing PCR and culture to be performed on the same sample. Monitoring which involves quantitation of infectious (culture) and total (PCR) HIV-1 in the same plasma sample will be expedited by use of this type of dual evaluation.

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