Diagnosis of Human Immunodeficiency Virus Infection Using Citrated Whole Blood

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Standard isolation of human immunodeficiency virus type 1 (HIV-1) from peripheral blood mononuclear cells (PBMC) requires 5 to 20 ml of blood, and the centrifugal separation of PBMC is expensive and time-consuming. Whole-blood coculture techniques use small sample volumes, do not require centrifugation, and allow measurement of the total viral burden in peripheral circulation. We compared the results of citrated whole-blood coculture with those obtained by the standard AIDS Clinical Trials Group PBMC semiquantitative culture method and reverse transcription-PCR quantitation of plasma HIV-1 RNA levels. PBMC cocultures were set up with added erythrocytes (RBCs) to determine if the presence of RBCs affects the replication of HIV-1 in vitro. The mean number of cells required for a p24-positive PBMC coculture was approximately seven times greater than that required for a positive citrated whole-blood coculture (P < 0.01). At volumes of 100, 50, and 25 μl, the sensitivities of the whole-blood coculture were 94.5, 93.6, and 87.3%, respectively. The PBMC culture in the presence of added RBCs was more sensitive than PBMC coculture alone. The citrated whole-blood coculture was simple to perform, produced a reliable diagnosis of HIV infection in adult volunteers, was more sensitive than previously reported techniques even in half the culture time, and showed less variability than the PBMC coculture. Citrated whole-blood coculture may be a useful and efficient tool for diagnosing infection with HIV-1.

Isolation of human immunodeficiency virus type 1 (HIV-1) from peripheral blood mononuclear cells (PBMC) is an accepted standard for diagnosing infection with HIV-1 (11, 16). The technique requires 5 to 20 ml of blood and is therefore less than ideal for use with infants, young children, and anemic adults (4). Further, the culture is expensive and time-consuming, because it requires the separation of PBMC from whole blood. These limitations spurred the development of microculture techniques to quantitate the viral load in PBMC and in the plasma of adults and children (1, 4, 10, 17). While using comparatively smaller volumes of blood, these methods still require isolation of PBMC by centrifugal separation.

Whole-blood culture techniques to isolate HIV-1 use small sample volumes, do not require the centrifugal separation of PBMC from samples, and allow measurement of the total viral burden in the peripheral circulation. Earlier studies using heparinized whole blood mandated 40 days for completed culture and isolated HIV-1 at a rate of only 20% (4). In other studies, heparinized whole blood was used successfully in an end point dilutional culture method for the diagnosis of vertical transmission of HIV-1 and in the diagnosis of HIV-1 infection in pediatric cases (2). However, previous research has raised questions about the use of heparin as an anticoagulant when culturing HIV-1 (3, 5). Heparin is known to interfere with PCR amplification, and it may inhibit the binding of laboratory amplification, and it may inhibit the binding of laboratory...
HIV-1-positive PBMC ranging in number between $1 	imes 10^4$ and $3.2 	imes 10^5$ in serial fivefold dilutions set up in duplicate. The cultures were maintained for 14 days. On day 7, 1 ml of culture supernatant was replaced by fresh culture medium containing $5 	imes 10^5$ PHA-stimulated HIV-1 negative PBMC. Culture supernatant from day 14 was tested for HIV-1 p24 antigen by the viral quantity assessment standardized p24 enzyme immunoassay (ABBOTT, Abbott Park, Ill.) procedure. Retrovirus Laboratory Management Program software (Datawork, Seattle, Wash.) was used to calculate the viral load, which was expressed as infectious units per milliliter cells.

Whole-blood coculture. Cocultures containing $10^5$ HIV-negative PHA-stimulated PBMC, 1 ml were established in 1 ml of medium (RPMI 1640 medium with 10% heat-inactivated fetal bovine serum, 2 U of IL-2, 2 mM L-glutamine, and penicillin-streptomycin (100 U/ml-100 mg/ml) along with citrated whole blood from HIV-1-positive patients added at serial twofold volume reductions of 100, 50, 25, 12.5, and 6.5 μl. The samples were set up in quadruplicate. Cultures were maintained for 14 days. The cultures were refed with 0.5 ml of the fresh medium at an interval of 3 to 4 days. Culture supernatants from day 7 were sampled for p24 production by using Vironostica HIV-1 p24 antigen kits according to the manufacturer's instructions (Organon Teknika, Durham, N.C.).

PBMC coculture with RBCs. HIV-1 was cultured from infected PBMC with and without the addition of RBCs from HIV-1-seronegative donors. PBMC coculture with RBCs was previously described (18). Briefly, HIV-1-negative PBMC were stimulated with PHA (5 μg/ml) and IL-2 (2 U/ml) for 72 h and washed before coculture with PBMC from HIV-positive individuals. RBCs, obtained by passing the HIV-negative blood through a leukocyte removal filter (RXCL 1; Pall Biomedical, Inc., Fajardo, P.R.), were washed with RPMI 1640 and counted. Cocultures contained 1:5 and 1:10 ratios of infected to uninfected cells and 5 x $10^5$ RBCs. Cultures without RBCs were set up as controls. All cultures were refed with 0.5 ml of the fresh medium at an interval of 3 to 4 days. Culture supernatants from day 7 were sampled for p24 production by using Vironostica HIV-1 p24 antigen kits according to the manufacturer's instructions (Organon Teknika).

Plasma HIV-1 RNA measurement. Citrated plasma was stored at $-80^\circ$C for batched analysis of HIV-1 RNA (13) by Roche Amplicor HIV-1 Monitor Assay (Laboratory Corporation of America, Research Triangle Park, N.C.).

Statistical analysis. In both PBMC and whole-blood culture methods, the end point was defined as the mean of the lowest dilutions in which at least 50% of the cultures were p24 positive. The mean number of cells required to isolate HIV-1 from each sample was determined. For PBMC cocultures, the count was obtained by Sysmex Microcell counter (TOA Medical Electronics, Carson, Calif.). This number was then used to estimate the PBMC content of whole blood. In whole-blood coculture, each patient’s lymphocyte count/whole-blood volume ratio was calculated. In each sample, this ratio was used to obtain cell counts from known volumes of blood. Student’s t test was used to compare differences in the mean number of cells required for positive culture by each method. The sensitivity of the whole-blood culture method was assessed at 100-, 50-, and 25-μl volumes and compared with that of PBMC coculture at $1 	imes 10^6, 2 	imes 10^5$, and $4 	imes 10^4$ cells in culture. Data generated from cocultures with and without RBC were analyzed using a chi-square test for matched pairs data.

RESULTS

PBMC versus whole-blood coculture. A total of 55 blood samples were obtained from 9 HIV-1-seropositive volunteers during five to seven clinic visits. The mean number of cells required for virus recovery by the PBMC coculture method ($1.3 	imes 10^5 \pm 4.0 \times 10^2$) was approximately seven times greater than that required by the whole-blood coculture method ($1.7 \times 10^4 \pm 4.6 \times 10^3$). According to the two-tailed t test, the cell requirement in the PBMC coculture method was significantly greater than the cell requirement in the whole-blood coculture method ($P < 0.01$).

Figure 1 shows the mean number of cells required for an HIV-1-positive culture by visit. For the whole-blood coculture, consistently fewer cells were required and the standard error was uniformly narrow. In the PBMC coculture method, the number of cells required for virus isolation was greater, and the standard error was larger and varied widely. At volumes of 100, 50, and 25 μl, the sensitivities of the whole-blood coculture method were 94.5, 93.6, and 87.3%, respectively, while the sensitivities of PBMC cocultures at $1 \times 10^6, 2 \times 10^5$, and $4 \times 10^4$ cells were 94.5, 90.9, and 83.6%, respectively.

PBMC cocultures with and without RBCs. PBMC samples obtained from 23 HIV-1-seropositive patients (including the 9 patients used for the whole-blood culture) were cultured with and without unrelated donor RBCs for a total of 46 matched data pairs. Without the addition of RBC, 14 of 23 cultures (61%) were p24 positive. With the addition of RBC, 19 of 23 cultures (83%) were p24 positive. This difference was significant by the chi-square test for matched pair data ($P < 0.05$).

PBMC coculture was highly sensitive, required only 7 as opposed to 14 days, and a reliable diagnosis of HIV-1 infection in adult volunteers, was possible in 7 of 8 patients using the PBMC coculture method described above. This method allows small blood volumes to be handled, is simple to perform, and does not require separation of the blood samples into cellular subsets. The method produced a reliable diagnosis of HIV-1 infection in adult volunteers, was highly sensitive, required only 7 as opposed to 14 days, and showed less variability than the PBMC coculture method. One

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<th>TABLE 1. Mean viral loads of patients at visits 1 through 7</th>
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* Detected by reverse transcription-PCR Roche Amplicor Monitor Assay.
previous study reported a similar method of heparinized whole-blood culture with a sensitivity of 91.9% (9). The cited whole-blood culture presented here was even more sensitive, even though we used one-fifth the volume of blood.

In our PBMC coculture, HIV-1 isolation was facilitated by the addition of RBCs. A chemokine receptor that binds to many of the α and β chemokines has been identified on the surfaces of RBCs (6, 12, 14). Given the recent identification of antiviral chemokines (7, 15), the presence of the RBCs in the cocultures may enhance HIV-1 growth by removing the chemokines from the supernatant. Furthermore, infectious HIV-antibody-complement complexes may bind to the complement receptors on RBCs and contribute to the whole-blood viral inoculum. The free HIV-1 virions in plasma may contribute only to multiply the higher sensitivity of the whole-blood culture. Studies indicate that less than 1/1,000 plasma virions are infectious in vitro (11). Given the fact that strong positive cultures were always detected at 6 to 100 µl of whole-blood volumes containing ~1 to 30 µl of plasma, it is unlikely that our volunteers’ plasma, generally containing fewer than 10⁵ virions/ml, would contribute to positive results.

Efficient use of blood samples avoids repeated needle sticks of HIV-1-infected persons and may permit evaluation of infants, children, and anemic adults. Semiquantitative whole blood culture may be a useful tool in diagnosing HIV-1 infection in newborns and infants, in whom the presence of vertical transmission of human immunodeficiency virus type 1 and for titration of infected peripheral blood mononuclear cells. J. Clin. Microbiol. 28:734–737.

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REFERENCES