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Marked Differences in Quantity of Infectious Human Immunodeficiency Virus Type 1 Detected in Persons with Controlled Plasma Viremia by a Simple Enhanced Culture Method

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Culture of autologous CD4 lymphocytes from peripheral blood mononuclear cells compared favorably with two other methods for the measurement of cell-associated human immunodeficiency virus type 1 (HIV-1). For subjects with undetectable HIV-1 RNA levels in plasma, there was a 10,000-fold range of cell-associated virus detected. This method provides a simple and reproducible means for monitoring cell-associated HIV-1.

Quantifying cell-associated human immunodeficiency virus type 1 (HIV-1) in subjects with virus levels in plasma below the limit of detectability could help to predict early treatment failure or to stratify patients for therapeutic trials. Such quantification studies are difficult to perform because of their complexity, and the amount of blood that is required. We have developed an enhanced culture method that can accurately and efficiently quantify cell-associated virus from peripheral blood mononuclear cells (PBMC) of patients with suppressed plasma viral load and have compared this method with another method for culture of CD8-depleted PBMC using anti-CD3 and anti-CD28 stimulation (4, 5), as well as with a standard method used to culture HIV-1 from unseparated PBMC (2).

For the HIV-1 enhanced detection assay, PBMC were resuspended at a concentration of 10^6 cells/ml in RPMI 1640 medium (Sigma, St. Louis, Mo.) supplemented with 10% heat-inactivated fetal calf serum (Sigma), penicillin (50 U/ml), streptomycin (50 μg/ml), L-glutamine (2 mM), HEPES buffer (10 mM), and 100 U of recombinant human interleukin-2 (Hoffmann-La Roche, Nutley, N.J.) per ml. For qualitative assays, PBMC (at a concentration of 10^6 cells/ml) were cultured either in 24-well tissue culture plates (Becton Dickinson, Lincoln Park, N.J.) at 10^6 cells per well or in 25-cm^2 polystyrene flasks (T25) (Becton Dickinson) at 10^7 cells per flask. A CD3-CD8-bispecific monoclonal antibody (MAb), which selectively depletes CD8 cells while activating CD4 cells, was added at a final concentration of 1 μg/ml (6). Cell cultures were incubated at 37°C in a humidified 5% CO_2 atmosphere and maintained for a 21-day period with twice-weekly medium exchanges. Supernatants were collected weekly before the medium change for the measurement of HIV-1 p24 antigen by an enzyme-linked immunosorbent assay (DuPont, NEN Research Products, Boston, Mass.). For quantitative assays, six fivefold dilutions of PBMC were used (25 million to 8 thousand). The 25- and 5-million PBMC dilutions were cultured in flasks in duplicate, and the higher dilutions were cultured in 24-well plates in quadruplicate. The number of infectious units per million (IUPM) PBMC was calculated from the pattern of positive wells by the method of maximum likelihood (3).

After 2 weeks in culture, cells were collected for the measurement of CD4 and CD8 T-lymphocyte populations. Three-color staining for CD3, CD4, and CD8 expression with a CD4 fluorescein isothiocyanate-CD8 phycoerythrin-CD3 peridinin chlorophyll protein-conjugated MAb (Becton Dickinson, San Jose, Calif.) was analyzed by flow cytometry. CD8 lymphocyte depletion was 99.6% and >95% of the cells were $CD4^+$. Three-color staining with a CD3 peridinin chlorophyll protein-conjugated, CD4 fluorescein isothiocyanate-conjugated (Becton Dickinson), and CXCR4 or CCR5 (Pharmingen, San Diego, Calif.) MAb was performed on culture days 0 and 14 on samples from 13 HIV-1-infected subjects and 10 healthy donors.

Our method was compared to another one, namely the enhanced culture of CD8-depleted PBMC with CD4 stimulation using immobilized anti-CD3 and anti-CD28 antibodies (4, 5). This method was performed as described previously (4, 5) and according to a detailed protocol kindly provided by C. Spina, University of California, San Diego. We also compared our enhanced culture to a standard AIDS Clinical Trials Group (ACTG) quantitative unseparated PBMC microculture, as described previously (2). Our assay was able to detect virus in all seven specimens from patients with HIV-1 RNA levels in plasma of <200 copies/ml, using an input of 10 million PBMC, compared to the detection in two of seven samples with the ACTG coculture method ($P = 0.02$, Fisher's exact test) (Table 1). The other qualitative CD8 cell depletion method, involving cell manipulation and immobilized anti-CD3 and anti-CD28 antibody stimulation, recovered virus from three of five samples from patients with HIV-1 RNA levels in plasma of <200 copies/ml. The methods performed similarly with four specimens from patients with HIV-1 RNA levels in plasma of >200 copies/ml. These results were obtained with similar numbers of input cells used in the three assays.

The HIV-1 enhanced detection assay was reproducible. We compared IUPM obtained from assays done on fresh and on freeze-thawed cells from three patients. The variation between fresh and freeze-thawed cultures ranged from 0.4- to 2.8-fold (average, 1.46-fold; standard deviation [SD], 1.2-fold) (data not shown). We also compared specimens from three patients on stable therapy over two or three consecutive visits spanning 3 days to evaluate clinical variability. The first subject had IUPM of <0.01 on days 1, 2, and 3; the second had IUPM of 0.37 on day 1, 0.13 on day 2, and 0.32 on day 3; the third had

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TABLE 1. Comparison of three HIV-1 detection assays

Patient no.	HIV-1 RNA level in plasma (copies/ml)	Presence of HIV-1 p24 determined with MAb(s) and input cells[a]			ACTG PBMC culture result[e] with 10×10^6 PBMC
		Anti-CD3-CD8[b] and 10×10^6 PBMC	Anti-CD3-CD8[b] and 1×10^6 PBMC	Anti-CD3 and anti-CD28[c] and 2×10^6 $CD8^-$ cells	
1	30,000	+ (7)	+ (7)	+ (7)	+ (7)
2	29,930	+ (7)	+ (7)	+ (7)	+ (7)
3	2,415	+ (7)	−	+ (7)	−
4	1,440	+ (7)	+ (7)	+ (7)	+ (7)
5	<200	+ (14)	+ (7)	ND[d]	+ (22)
6	<200	+ (14)	−	+ (7)	−
7	<200	+ (14)	−	+ (22)	+ (7)
8	<200	+ (14)	+ (14)	+ (22)	−
9	<200	+ (14)	+ (14)	ND	−
10	<200	+ (20)	−	−	−
11	<200	+ (22)	−	−	−

[a] Detected by enzyme-linked immunosorbent assay. Values in parentheses indicate the number of days after the start of culture. Cultures ended at 21 days.
[b] CD8 T-cell depletion and CD4 cell stimulation were achieved by the bispecific MAb CD3-CD8.
[c] CD8 T-cell depletion and CD4 cell stimulation were achieved by immobilized anti-CD3 and anti-CD28 MAbs.
[d] ND, not done.
[e] Differences between CD3-CD8 and ACTG cultures were significant ($P = 0.02$, Fisher's exact test). Values in parentheses indicate the number of days after the start of culture.

IUPM of 0.65 on day 1 and 0.13 on day 3 (a culture was not available for day 2). The variation among IUPM ranged from 0 to 5-fold (average, 2.6-fold; SD, 2.5-fold). There were no significant differences (1.02-fold increase; SD, 0.37-fold) in the percentages of CD4 lymphocytes expressing CXCR4, and there was an average of a 2.13-fold (SD, 1.32-fold) increase in CCR5 expression after stimulation with the CD3-CD8 MAb for the samples from HIV-1-infected subjects (data not shown).

We next studied PBMC from 22 patients who had been on highly active antiretroviral therapy for prolonged periods, using the quantitative version of our assay (Table 2). Virus was recovered from 18 of 22 patients with an input of 66 million cells. We repeated the assay for two of the negative cultures, using more cells (125 million PBMC), and one of the two was virus positive at the higher PBMC concentration. For the 15 patients with HIV-1 RNA levels in plasma below 50 copies/ml, the number of input PBMC necessary to generate a positive culture ranged from 8 thousand to >25 million, which translated into a wide range of IUPM (from <0.01 to 44.57).

Two patients, one with acute and the other with chronic HIV-1 infection, had their treatments interrupted after virus was suppressed to below 50 copies/ml for 18 and 21 months, respectively. For both, HIV-1 RNA levels in plasma rebounded quickly. For one patient, IUPM increased from <0.06 to 0.22 before RNA in plasma became detectable, and for the other, IUPM increased from 0.23 to 3.27 at the time RNA in plasma became detectable.

The enhanced culture method is sensitive and easy to perform. The sensitivity can be explained by the ability of the CD3-CD8-bispecific MAb to deplete the CD8 lymphocytes and to preferentially activate the CD4 lymphocytes to proliferate via CD3 stimulation. The CD8 cell depletion is almost complete (<0.01% remaining at day 14 of culture) (data not shown). The CD3-CD8-bispecific MAb stimulation allows the maintenance of cultures for at least 21 days without further addition of antibody or feeder cells. This one-step process makes the method relatively simple to use in comparison to methods requiring physical separation of cells or cell sorting in a flow cytometer. Our assay requires only 40 to 60 ml of blood, in contrast to some other assays which may even require leukapheresis (1). Although this culture method does not specifically deplete activated CD4 lymphocytes or target resting CD4 lymphocytes, it quantitates the amount of replication-competent virus in PBMC, as do other methods for culturing CD8-depleted cells without initial separation of resting cells (4, 5). This measurement reflects both any persistent replication and the remaining latent reservoir in blood, including cells harboring both integrated and unintegrated HIV-1.

There was an interpatient variation of approximately 4 $\log_{10}$ in IUPM, indicating that individuals with suppressed plasma viremia can have widely different levels of replication-competent HIV-1 within their cellular reservoirs and should not be considered a uniform group. It may be possible to stratify patients with undetectable HIV-1 RNA levels in plasma on the basis of the amount of residual cell-associated replication-competent virus present in order to evaluate different treatment strategies and predict prognosis. Although our data are insufficient to document the predictive value of this assay,

TABLE 2. Quantitative analysis of cell-associated HIV-1

Patient no.	Length of time of therapy (wks)	Treatment regimen[a]	HIV-1 RNA level in plasma (copies/ml)	IUPM in PBMC
13	8	ZDV/3TC/IDV	12,400	16.82
18	83	ZDV/3TC/IDV	9,606	0.11
16	14	ZDV/3TC/IDV	<200	9.26
19	82	ZDV/3TC/IDV	<200	0.11
14	84	ZDV/3TC/IDV	<200	0.02
8	56	ZDV/3TC/IDV	<50	3.28
5	60	ZDV/3TC/IDV	<50	3.27
17	82	ZDV/3TC/IDV	<50	3.27
4	60	ZDV/3TC/IDV	<50	1.83
9	72	ZDV/3TC/IDV	<50	1.13
2	48	ZDV/3TC/IDV	<50	1.08
7	60	ZDV/3TC/IDV	<50	0.07
1	48	ZDV/3TC/IDV	<50	0.04
6	68	ZDV/3TC/IDV	<50	0.01
3	68	ZDV/3TC/IDV	<50	<0.01
12	68	ZDV/3TC/NFV	<50	0.04
11	56	ZDV/3TC/NFV	<50	<0.01
10	6	D4T/3TC/NFV	17,300	16.82
20	12	D4T/3TC/NFV	<50	<0.01
22	96	D4T/3TC/IDV	<50	44.57
21	84	D4T/3TC/IDV	<50	0.48
15	16	D4T/3TC/IDV	<400	0.07

[a] ZDV, zidovudine; 3TC, lamivudine; IDV, indinavir; D4T, stavudine; NFV, nelfinavir.

further studies will evaluate this possibility and should provide us with a better understanding of the complex interactions among virus reservoirs, immune responses, antiretroviral therapy, and long-term prognosis.

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