Sensitive Reverse Transcriptase Assay To Detect and Quantitate Human Immunodeficiency Virus

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A sensitive biochemical assay of viral reverse transcriptase (RT) was developed that is useful for both the detection and quantitation of human immunodeficiency virus (HIV), the agent responsible for acquired immune deficiency syndrome in humans. This assay gave a 20- to 40-fold increase in enzyme activity over the current method used for RT detection of HIV. The test is based on a previous biochemical study showing the unusual stability of avian oncornavirus RNA-dependent DNA polymerases at 30°C for at least 2 days. Our study shows that the HIV polymerase is stable at 30 to 37°C for up to 3 days. By using this sensitive RT assay, as few as 250 HIV virions can be quantitated directly in tissue culture medium. This assay should prove useful in studies in which the detection of HIV or the quantitation of the number of virions is required.

The study of human immunodeficiency virus (HIV), the agent associated with acquired immune deficiency syndrome (AIDS), is hampered by difficulty in detecting and quantitating the virus. HIV is present at very low levels in the blood and body fluids of infected individuals and requires 2 to 3 weeks of *in vitro* cell culture before a detectable amount of virus is available for testing (1, 7, 10). Numerous tests have been used to detect HIV at these low levels. Among the biochemical tests used are the reverse transcriptase (RT) assay (RTA) to detect viral polymerase (16) and *in situ* hybridization to directly detect viral nucleic acid in infected cells (20). A wide array of immunological procedures has been adapted for virus antigen-antibody detection. Among these are indirect immunofluorescent-antibody technique (4), enzyme-linked immunosorbent assay (3), radioimmunoassay (3), Western blotting (immunoblotting) of viral proteins (19), dot immunoblotting (2), and the antigen-capture enzyme-linked immunosorbent assay (12). There is currently no clear consensus as to the best method for HIV detection, as each method offers some advantages.

The quantitation of HIV has relied on virological methods of direct particle counts either by electron microscopy (13) or by biological assays, such as plaque titration (8) and 50% tissue culture infective dose determination (12). Unfortunately, electron microscopic counts and 50% tissue culture infective dose determinations are technically laborious and are therefore not useful for routine quantitation of a large number of samples. The plaque titration of HIV has also proven difficult to do, and most laboratories have not been successful in using this procedure. Thus, studies for which determination of virus number would be informative have been hindered.

Previously, Tereba and Murti (22) have reported on the development of a very sensitive RTA for avian oncornaviruses. They exploited the unique stability of the viral polymerase at 30°C for assaying enzyme activity in a 24-h period. Since DNA was synthesized at a linear rate, this sensitive assay was reliable for the detection of as few as 560 virions. In this report, we also describe the stability of the HIV polymerase at 30 to 37°C and the adaptation of this sensitive RTA for the detection of as few as 250 HIV particles. Thus, this sensitive RTA may prove useful in

overcoming the present difficulties in detecting and quantitating HIV and allow better virological studies.

MATERIALS AND METHODS

Cell and virus culture. The established CEM T-cell line, derived from the peripheral blood of a child with acute leukemia (6), was used to grow the HIV isolate human T-cell lymphotropic virus type III (HTLV-III) (7). The virus was used directly from clarified culture medium supernatant or was concentrated by precipitation with half the volume of 30% polyethylene glycol (PEG)-0.4 M NaCl. The virus precipitate was collected by centrifugation, solubilized in a buffer containing 0.025% Triton X-100, and stored at -20°C (17). Peripheral mononuclear cell (PMC) cultures from homosexual males in the Multicenter AIDS Cohort Study were prepared by established procedures of cocultivation with normal donor PMCs, and culture supernatants were assayed directly or precipitated with PEG for detection of HIV by RTA (10, 14).

All cells were maintained in RPMI 1640 medium supplemented with 20% fetal bovine serum (FBS) and 10% interleukin-2 and cocultivated with fresh normal donor PMCs (2×10^6) every 3 to 4 days to enhance virus replication.

RTA. Polymerase assays were done at 30 to 37°C by mixing 50 μ l of virus sample preparation with 45 μ l of a $2\times$ reaction buffer described by Hoffman et al. (9) and consisting of 50 mM Tris hydrochloride, pH 7.9, 5 mM dithiothreitol, 0.3 mM reduced glutathione, 5 mM $MgCl_2$, 150 mM KCl, 0.5 mM ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid, 0.05% Triton X-100, 2% ethylene glycol, and virus that had been solubilized for 15 min. The virus-buffer mixture (95 μ l) was added to an autoclaved glass culture tube containing 5 μ l of template primer [poly(rA) · oligo(dT) or poly(dA) · oligo(dT)] and 20 μ Ci of [3H]dTTP, and the enzyme reaction was allowed to proceed until the designated time. The reaction was stopped by addition of 1 ml of cold 10% trichloroacetic acid containing 0.02 M sodium PP_i , and the DNA product was allowed to precipitate for 2 h on ice. The precipitate was collected onto a Whatman GF/A glass fiber filter, rinsed well with 5% trichloroacetic acid and absolute ethanol, and dried, and the amount of radioactivity was determined by counting in a liquid scintillation spectrometer.

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FIG. 1. Kinetics of RT activity at 30 and at 37°C. The rate of incorporation of [³H]dTMP during RTA for HIV was determined, as described in Materials and Methods, for 1 to 24 h (A) and 1 to 6 days (B).

Electron microscopy. The preparation of virus and its examination by negative staining has been described previously (18). Briefly, 20 ml of cultured virus supernatant was passed through a Millipore filter (pore size, 0.22 μ m), and the virus in the filtrate was precipitated with PEG and collected by centrifugation. The virus pellet was suspended in 0.2 ml of phosphate-buffered saline and centrifuged at 1,000 $\times$ *g* for 30 min at 4°C to remove any aggregates. Three virus dilutions (1:2, 1:10, and 1:20) were prepared with 2% paraformaldehyde for counting. A 0.5- μ l sample of virus dilution was spread carefully onto a carbonized collodion membrane on a 300-mesh copper grid with a micropipetter, and then 0.5 μ l of 2% uranyl acetate (ethanol solution) was placed on the same grid. The virus samples were immediately observed with a Hitachi H-600 electron microscope.

Triplicate samples for each virus dilution were examined with an electron microscope, and virus-like particles were counted in five meshes (area of each mesh was 3.0 $\times$ 10³ μ m²). The number of virus particles over the entire area of the grid was calculated and used to determine the number of virus particles per milliliter in the concentrated preparation.

Reagents. The chemicals employed for the RTA were obtained from Sigma Chemical Co. or Boehringer Mannheim Biochemicals. The template primers were purchased from Pharmacia, Inc., and suspended in sterile distilled water at 2.5 μ g (0.05 U) in 5 μ l. The [³H]dTTP (specific activity, 50 to 80 Ci/mmol) was from New England Nuclear Corp. or ICN Radiochemicals as a 50% ethanol solution, and aliquots were evaporated to dryness under vacuum in a glass culture tube just before use.

RESULTS

Kinetics of RTA. An HIV isolate (HTLV-III) was grown in CEM cells and served as the source of RT for our studies. We were also able to grow HIV in other continuous T-cell lines (MOLT 4 and HUT-78) and in normal donor PMCs, as reported by others (1, 7, 10). The virus grown in these different cell cultures was readily detected, at the same level, by the RTA reported here (data not shown).

The polymerase reaction of HIV was examined for a longer reaction time to determine if higher levels of activity could be achieved to improve virus detection. The kinetics of the RT reaction for HIV at 30 and 37°C in a 24-h period is

shown in Fig. 1A. The reaction at both temperatures showed linear kinetics for at least 12 h with only slightly decreasing kinetics thereafter. The reaction yielded greater activity at 37°C but with slightly less linear kinetics compared with the reaction at 30°C. The polymerase was quite stable in the reaction buffer, as it remained active at a near-linear rate over a 3-day period of time (Fig. 1B). We next examined the heat stability of the virus (and the enclosed polymerase) in RPMI medium. Identical virus samples were incubated at 30, 37, and 45°C for 0 to 3 h, and their RT activity was determined. The virus polymerase was quite stable in RPMI medium at 30°C and was only slightly inactivated at 37°C (Fig. 2). In contrast, the enzyme was inactivated more rapidly at the elevated temperature of 45°C, with about one log activity decrease after 30 min.

The samples that we examine for HIV by the RTA are directly from cell culture supernatants or a PEG concentrate of culture supernatant. We thus needed to determine if constituents of these samples interfere with the RTA. The FBS in RPMI medium may affect enzyme activity, and so we assayed identical HIV preparations in RPMI medium with various concentrations of FBS. We found that medium with 2.5% or less FBS had no effect on the polymerase assay, but increasing amounts above this level yielded decreasing levels of activity (Fig. 3A). We routinely concentrate the PMC culture supernatants by PEG precipitation to detect small amounts of HIV. It has been reported that PEG inhibits the RT activity of HIV (9), and we also found this to be true. In our RTA, we have been testing 10 μ l of a 20-fold viral concentrate by PEG precipitation of 4 ml of culture supernatant. It is very difficult to obtain viral precipitate which is free from the PEG in solution. To test the effect of this contaminating PEG on enzyme activity, we added increasing amounts of a PEG-precipitated concentrate from 4 ml of normal PMC culture supernatant to identical HIV preparations in 100 μ l of assay buffer. The PEG concentrate was shown to interfere with RT activity, as increasing amounts (1 $\times$ to 5 $\times$) caused the level of activity to drop from 80 to 43% (Fig. 3B).

Sensitivity of RTA. The number of HIV virions in a concentrated stock solution was determined by a direct particle count of negative-stained virus with the electron

FIG. 2. RT activity of heat-treated HIV in RPMI culture medium kept at 30, 37, and 45°C for 0.5 to 3 h. The RTA for the amount of [³H]dTMP incorporated is described in Materials and Methods.

FIG. 3. Effect of two constituents on RT activity of HTLV-III. Identical solubilized virus samples in RPMI culture media with increasing percentages of FBS (A) or increasing amounts of the PEG precipitate solution ($1\times = 10\ \mu\text{l}$ of a 20-fold concentrate of 4 ml of donor PMC culture supernatant, $2\times = 20\ \mu\text{l}$, etc.) were assayed for RT activity, as described in Materials and Methods.

microscope. The virus was prepared from infected CEM T-cell culture supernatant. The virus suspension was counted at three different dilutions to minimize error associated with nonuniform distribution of virus particles caused by clumping or unequal drying. The particle counts for HIV are presented in Table 1. For each virus dilution, three grids were counted (number of virus particles in five meshes of the grid). The mean value of the three samples was calculated to be 2.5×10^7 virus particles per ml in the concentrated preparation.

The sensitivity of the polymerase assay was determined by assaying serial dilutions of the stock HIV at 30°C in a 22-h period. The enzyme activity was linearly related to the number of virus particles (slope of 1.1 for full logarithmic plot) (Fig. 4). Therefore the number of virus particles is directly correlated with, and can be estimated from, its polymerase activity. The amount of radioactivity for 2.5×10^2 to 7.5×10^4 virus particles ranged from 1.0×10^4 to 6.3×10^6 cpm during a 22-h assay. The background radioactivity of an uninfected PMC preparation over this same time period was 0.4×10^4 cpm. This assay is thus capable of detecting as few as 250 virus particles, with a polymerase activity that is greater than twice that of background levels of radioactivity.

Use of RTA for HIV detection and quantitation. This sensitive RTA (18 h, 37°C) was used for HIV detection in 50 μl of culture supernatant from PMC cultures of volunteers in the Multicenter AIDS Cohort Study and then compared with the more widely used assay (1 h, 37°C). The results of six culture determinations are presented in Table 2. As expected, the 18-h assay consistently showed higher levels of activity than did the 1-h assay in the presence of viral RT. The level of background radioactivity did not increase with the longer incubation time, as negative-control cultures had the same levels (cultures no. 1 and 2). If virus was present in larger numbers, the two assays offered the same level of detection (cultures no. 3 and 4). However, if virus was present in low numbers, it was detected by the 18-h assay but was missed by the 1-h assay (cultures no. 5 and 6).

The sensitivity of the RTA for various retroviruses from this and previous studies is presented in Table 3. It shows that different retroviruses have various levels of detection by RTA. The widely used assay (1 h, 37°) at best, had a detection level on the order of 10^4 virus particles. The sensitive RTA had a virus detection level on the order of 10^2

TABLE 1. Determination of HIV virion concentration^a

Dilution	Grid no.	No. of virions/mesh	Virion concn ^b (10^7 virions/ml)
1:2	1	21.8	2.6
	2	25.6	
	3	27.4	
Mean		24.8	
1:10	1	6.4	3.0
	2	5.8	
	3	5.4	
Mean		5.9	
1:20	1	1.4	1.8
	2	1.6	
	3	2.4	
Mean		1.8	

^a Three grids for each virus dilution were counted with a Hitachi H-600 electron microscope. Five meshes per grid were examined, virus-like particles were counted, and the mean number of particles was calculated.

^b The formula for quantitating the concentration of virions in a 0.5- μl sample of the 1:2 dilution was as follows: (number of virions per mesh) (area of grid/area of mesh) (sample volume dilution factor per grid) (virus preparation dilution factor) = $(24.8\text{ virions}) (7.85 \times 10^5\ \mu\text{m}^2/3.025 \times 10^3\ \mu\text{m}^2) (1,000\ \mu\text{l/ml})/(0.5\ \mu\text{l}) = 2.6 \times 10^7$ virions per ml. Other quantities were calculated accordingly. Mean of all values, 2.5×10^7 virions per ml.

virus particles. Among the retroviruses studied so far, HIV is the most sensitive to detection by its RT activity. As few as 250 virions can be detected by this type of assay.

DISCUSSION

Tereba and Murti (22) were the first to describe the stability of the RNA-dependent DNA polymerase of various avian retroviruses. They exploited this finding in a RTA at 30°C for 24 h that greatly increased the detection of virus. Our study shows that the polymerase of HIV is also stable for a long period of time, giving higher levels of DNA product that allow greater detection of virus. The viral RT is quite stable at 37°C , which is unexpected as enzyme activity usually decreases at this temperature after a period of time because of heat inactivation. This stability of the polymerase

FIG. 4. Sensitivity of RTA for HIV. Dilutions of a known concentration of virus (determined by electron microscopy) were made in RPMI culture medium, and its RT activity was measured as described in Materials and Methods at 30°C in a 22-h period.

TABLE 2. Detection of HIV in PMC culture supernatant by RTA^a

Culture no. and day of collection ^b	Incorporation of [³ H]TMP (10 ⁴ cpm) at 37°C			
	1-h assay	Culture ^c	18-h assay	Culture
1 (Donor PMC)	0.4	—	0.3	—
2				
7	0.4	—	0.7	—
14	0.4	—	0.4	—
21	0.4	—	0.7	—
3				
7	0.5	—	0.6	—
14	4.3	+	32.4	+
21	22.2	+	180.0	+
4				
14	0.9	—	0.6	—
21	0.6	—	1.5	+
28	1.6	+	4.0	+
5				
7	0.2	—	1.0	+
14	0.4	—	1.1	+
21	0.4	—	2.3	+
6				
14	0.9	—	1.1	+
21	0.8	—	1.9	+
28	0.5	—	1.2	+
Control HTLV-III	29.2	+	450.0	+

^a Samples of 50 µl of culture supernatant were assayed for RT activity, as described in Materials and Methods. Cultures were PMCs from a blood bank donor and from five healthy homosexual men participating in the Multicenter AIDS Cohort Study. Culture no. 2 was from an individual who was seronegative, while the remaining cultures were from individuals who were seropositive for HIV antibody by the enzyme-linked immunosorbent assay.

^b Day on which PMC culture supernatant of patient was collected for assay.

^c Culture is considered positive for the presence of RT if activity, as measured by incorporation of [³H]TMP into DNA, is at least 2.5 times greater than that of the normal donor PMC culture. Since this background level can vary from 3,000 to 4,000 cpm, we have set >10,000 cpm as the criterion for a positive result.

may be due in part to the assay reaction buffer, which contains ethylene glycol as a stabilizing agent. However, we have observed the polymerase in its encapsidated whole virus form to be also quite stable in only RPMI medium; it was not substantially inactivated at 30 to 37°C for 3 h but was inactivated at 45°C. This indicates that the HIV polymerase itself is quite heat stable. This finding corresponds well with reports on the loss of HIV infectivity at an elevated temperature of 56°C after 10 to 30 min but loss of infectivity at 37°C occurred only after a long period of 11 to 15 days (15, 21). Therefore, on the whole, the proteins of HIV appear to be quite stable at 37°C.

The RT reaction of HIV proceeds at a linear rate for at least 22 h at 30°C and so can be used for quantitation of the number of virus particles. This sensitive RTA may thus be useful in virus studies for both virus detection and an estimate of the number of viral particles present. This quantitation by RTA has the advantage of being less difficult and more rapid than are biological assays, such as 50% tissue culture infective dose determination or plaque titration. Since the polymerase is also less sensitive to heat, it has the added advantage of detecting virus in studies when biological infectivity may be decreased.

This study shows the usefulness of a longer RTA period in

TABLE 3. Sensitivity of RT for various retroviruses

Assay and virus ^a	No. of virions detected	Reference
1 h, 37°C		
MuMTV	2.6×10^4 – 6.7×10^4	Fine et al. (5)
AMV	2.5×10^4	Liebes et al. (11)
MuLV	8.8×10^4	Liebes et al. (11)
HIV	8.5×10^{3b}	McDougal et al. (12)
18–24 h, 30°C		
RSV	5.6×10^2 – 1.5×10^4	Tereba and Murti (22)
RAV	8.3×10^2	Tereba and Murti (22)
RNPV	2.1×10^3	Tereba and Murti (22)
GOPV	3.0×10^4	Tereba and Murti (22)
REV-T	3.0×10^6	Tereba and Murti (22)
HIV	2.5×10^2	Present study

^a Virus abbreviations: MuMTV, murine mammary tumor virus; AMV, avian myeloblastosis virus; MuLV, murine leukemia virus; RSV, Rous sarcoma virus; RAV, Rous-associated virus; RNPV, ringnecked pheasant virus; GOPV, golden pheasant virus; REV-T, reticuloendotheliosis virus, strain T.

^b Calculated value based on the estimate of 1.5×10^5 cpm = 10^4 50% tissue culture infective doses = 10^4 PFU = 10^8 virus particles.

detecting low levels of HIV in samples that would not be detected by the more routinely used 1-h assay. If large amounts of HIV are present, it can be conveniently detected directly in 50 µl of culture supernatant without any further processing. On the other hand, if small amounts of HIV are present, then concentrating the virus in the sample is required for its detection. We confirm the previous findings that FBS or PEG interferes with the polymerase reaction. Optimum detection of virus by the RTA would be in a sample that does not contain these constituents. Hoffman et al. (9) have recommended concentrating HIV by ultracentrifugation in a microcentrifuge. We have also found this to work well and routinely use this method now in our HIV studies.

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