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Quantitative RNA and DNA Gene Amplification Can Rapidly Monitor HIV Infection and Antiviral Activity in Cell Cultures

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We have developed a quantitative gene amplification procedure to assess the replication of human immunodeficiency virus (HIV) in cell cultures and evaluate the effect of drugs on viral replication. Increases in HIV *gag* RNA and DNA in phytohemagglutinin-stimulated normal peripheral blood mononuclear cells (PBMC) infected with HIV at very low multiplicity of infection paralleled the production of HIV p24 antigen in culture supernatants. Quantitative gene amplification was able to monitor the accumulation of viral nucleic acids in control cultures and demonstrate the effect of various concentrations of azidothymidine (AZT) on the replication of both AZT-sensitive and -resistant strains of HIV. The sensitivity of patient-derived virus strains to AZT could also be successfully measured by these procedures. The results of our studies suggest that quantitative measurement of HIV *gag* RNA and DNA can be used to monitor the kinetics of viral replication, antiviral activity, viral drug resistance, and mechanism of drug action.

Measuring the replication of human immunodeficiency virus (HIV) in vitro is essential for assessing the infectivity of various specimens and in examining the potency of compounds that may inhibit viral replication. Secreted structural proteins such as p24 antigen or the enzymatic activity of viral reverse transcriptase^(1,2) have been widely used to assess viral replication in vitro. However, recent reports have demonstrated that in certain situations a dissociation between integration, translation, and transcription of viral nucleic acids can occur.^(3,4) In evaluating the effect of antiviral agents on HIV, the presence or accumulation of viral nucleic acids should be examined in addition to structural or enzymatic markers to assess fully the activity of an antiviral compound and examine the mechanism by which it acts.

The presence of HIV nucleic acids in cells has primarily been measured directly by in situ hybridization or Southern blot analysis.⁽⁵⁻⁷⁾ With the application of the polymerase chain reaction (PCR) to assess the presence of HIV,^(8,9) very small amounts of HIV-specific nucleic acids can be detected. Recent studies have used quantitative gene amplification methods to measure proviral copy number in patient peripheral blood mononuclear cells (PBMC).⁽¹⁰⁻¹²⁾ We have applied a quantitative PCR technique to examine the kinetics of HIV replication in vitro and to assess the effect of antiviral drugs on the accumulation of HIV *gag* RNA and DNA. Our results suggest that this technique may be useful in

assessing infectivity, antiviral activity, viral resistance, and mechanism of drug action in an in vitro assay system.

MATERIALS AND METHODS

Cells and Viruses

PBMC from healthy HIV seronegative donors were isolated on Ficoll-Hypaque gradients and washed twice with phosphate-buffered saline (PBS, pH 7.2). The isolated PBMC were cultured for 3 days in complete medium (RPMI + 15% fetal bovine serum + 5% IL-2 + 1% penicillin-streptomycin) containing 3–5 $\mu\text{g/ml}$ phytohemagglutinin (PHA). The cells (PHA blasts) were then washed twice and resuspended in complete medium at 1×10^6 cells/ml.

Stocks of HIV strains IIIb and strain 691⁽¹³⁾ (kindly provided by D. Richman) were prepared by infecting PHA blasts with virus stocks and harvesting the supernatant 4–14 days later. For production of patient virus stocks, blood was collected with informed consent and the PBMC isolated over Ficoll-Hypaque gradients. The patient PBMC were then cocultured with an equal number of PHA blasts in complete medium for 4–14 days. The supernatants were collected and stored at -70°C , and titered in PHA blasts prior to use in experiments. One tissue culture infectious dose (TCID) was defined as the highest 10-fold dilution of virus stock added to 1×10^6 PHA blasts that yielded a positive p24 test (greater than 25 pg/ml) in the culture supernatant after 7 days.

HIV Cultures

Approximately 2×10^7 PHA blasts were incubated in 20 ml of complete medium containing various concentrations of AZT for 4–6 hr in 50-ml centrifuge tubes. HIV was then added to the cultures and incubated for 1–2 hr at 37°C. The cells were then washed three times with PBS, and resuspended in approximately 20 ml of complete medium containing the appropriate amount of drug. The cells were cultured in 25-cm² flasks for the duration of the experiment.

Sampling Procedures

At each sampling time point, the cells in each culture were thoroughly resuspended and 5 ml of the culture was removed. The culture sample was centrifuged for 5 min at 500g; the supernatant was removed and stored at -20°C for p24 assay, and the cell pellet was resuspended in 15 ml of PBS and centrifuged as before to remove extracellular virus particles. The supernatant was discarded, and the pellet was resuspended in 1 ml of PBS and transferred to a 1.5-ml microcentrifuge tube. The cells were again pelleted at 6000g for 30 sec, the supernatant removed, and the pellet resuspended in 275 μ l of PBS. The cell suspension was then divided into two aliquots: 200 μ l was removed and placed into another 1.5-ml microcentrifuge tube containing 200 μ l of 5 M guanidine isothiocyanate for later RNA extraction, and the remaining 75 μ l was diluted to 200 μ l with PBS for later DNA extraction. Both cell samples were stored at -70°C until the end of the experiment.

p24 Antigen Measurement

The amount of HIV p24 antigen in the supernatant of each culture was determined using a DuPont p24 antigen kit. The assay was performed according to manufacturer's instructions.

Nucleic Acid Purification

RNA was purified by the method described by Chomczynski and Sacchi.⁽¹⁴⁾ Briefly, 15 μ l of 3 M sodium acetate was added to each 200- μ l cell sample stored in guanidine isothiocyanate. The sample was then extracted with 200 μ l of water-saturated phenol and 40 μ l of

chloroform-isoamyl alcohol (49:1). After being held on ice for 20 min, the samples were centrifuged at 6000g for 20 min at 4°C. The aqueous layer was transferred to a clean microcentrifuge tube and the RNA was precipitated with 500 μ l of isopropanol for 1 hr at -20°C. The RNA was pelleted and resuspended in 200 μ l of guanidine. The RNA was precipitated again with 200 μ l of isopropanol for 1 hr at -20°C, pelleted, and washed once with 200 μ l of 75% ethanol. The final RNA pellet was then dried and resuspended in 10–20 μ l of diethylpyrocarbonate-treated water, and the amount of RNA in each sample was quantitated by spectrophotometry. Preliminary experiments demonstrated that specimens extracted by this method were free of contaminating DNA (data not shown).

DNA was isolated from cell pellets by digestion with proteinase K buffer (0.1 mg/ml proteinase K, 10 mM Tris-HCl, pH 8.3, 2.5 mM MgCl₂, 50 mM KCl, and 0.45% Tween-20) overnight at 55°C. The samples were then heated to 95°C for 5 min, and the DNA was precipitated by the addition of 500 μ l of absolute ethanol. After 1 hr at -20°C, the DNA was pelleted, washed once with 200 μ l of 75% ethanol, and pelleted again. The DNA pellets were dried and resuspended in 20–50 μ l of water, and the amount of DNA in each sample was quantitated by spectrophotometry.

Gene Amplification

RNA samples were reverse-transcribed prior to PCR amplification. One microgram of RNA was incubated with 10 pmoles of random hexamers, 0.5 mM of each dNTP, 2.5 units of RNasin, and 5 units of murine leukemia virus (MuLV) reverse transcriptase (Bethesda Research Labs) in 10 μ l of amplification buffer (10 mM Tris HCl (pH 8.3), 2.5 mM MgCl₂, 0.02% gelatin, and 50 mM KCl) for 30 min at 42°C, then at 95°C for 5 min.

PCR amplification was performed on the reverse-transcribed RNA samples and on 1 μ g of each DNA sample. Each reaction mixture contained the nucleic acid sample, 50 pmoles of primers SK39 and biotinylated SK38 (Synthetic Genetics, San Diego, CA), 200 μ M each of dNTP, and 2.5 units of AmpliTaq DNA polymerase (Perkin-

Elmer Cetus) in 100 μ l of amplification buffer. All samples were then overlaid with approximately 50 μ l of light mineral oil and amplified for 30 cycles on a Perkin-Elmer Model 480 with the following parameters: 95°C for 10 sec, 55°C for 30 sec, and 72°C for 30 sec, followed by a 10-min extension step at 72°C.

Quantitation of PCR Product

A nonisotopic enzyme-based assay was performed to quantitate the amount of amplified DNA in each sample. Microtiter plates were coated with 100 μ l of a 0.1 mg/ml avidin solution prepared in 0.05 M sodium carbonate (pH 9.6). After overnight incubation at room temperature, the avidin solution was discarded and the wells were filled with a blocking solution consisting of 5 \times Denhardt's solution, 1% gelatin, and 0.25 mg/ml herring sperm DNA. After at least 18 hr at 4°C, the blocking solution was discarded and 65 μ l of hybridization solution (5 \times SSPE, 5 \times Denhardt's solution) containing 1 pmole of horseradish peroxidase (HRP)-labeled SK19 was added to each well.

Immediately prior to assay, the PCR reaction products were heated to 95°C for 5 min, then rapidly cooled to 4°C. Five microliters of each PCR reaction product was then added to each well and the plate was incubated for 1–2 hr at 42°C to allow for capture and hybridization. The plate was then washed 20 times with 300 μ l of PBS containing 0.05% Tween-20. The HRP substrate *o*-phenylenediamine was prepared at 0.6 mg/ml in 0.1 M citrate buffer (pH 5.5), and 150 μ l of this solution was added to each well. After 10 min at room temperature, the reaction was stopped with 100 μ l of 1 N sulfuric acid, and the optical density of each well was measured at 490 nm. The log DNA or RNA copy number for each sample was then determined by comparing the optical density value for each sample to a standard curve generated from parallel amplification of dilutions of ACH2 cell DNA⁽¹⁵⁾ or an RNA *gag* gene construct.⁽¹⁶⁾

RESULTS

Quantitation of PCR Product

The use of biotinylated primers during the gene amplification step allowed

the use of a microtiter plate-based detection system.⁽¹⁵⁾ Avidin attached to microtiter wells captured the biotinylated PCR product, which could then be quantified using a HRP-labeled probe. The number of input HIV *gag* RNA or DNA copies in each PCR reaction was determined from standard curves generated from serial dilutions of ACH2 cell DNA or an RNA *gag* construct. A strict log-linear relationship existed between the copy number and the optical density in this assay system such that approximately 30–50,000 copies could be accurately measured. The range of sensitivity in this assay is similar to that seen with methods using radiolabeled probes such as dot blots or Southern blots.^(17–19) In these experiments, single RNA or DNA samples from each culture were amplified because our previous work^(15,16) showed a high reproducibility between replicate samples in this assay system.

Detection of HIV Replication and Drug Effect

We initially used laboratory-adapted strains of HIV to assess the ability of quantitative PCR to measure HIV replication and the effect of AZT on viral growth. Preliminary experiments examining the effect of inoculum size indicated that 10–100 TCID were sufficient to provide measurable p24, RNA, and DNA signals in a 7-day period that could be used effectively to compare the effect of various concentrations or AZT on replication (data not shown). In addition, while quantitative values (e.g., p24 antigen concentration on DNA copy number) were different when isolates were cultured on different weeks (primarily due to differences in PHA blasts), the overall relationship between the control and the drug-treated cultures was consistent on a week-to-week basis (data not shown).

For drug sensitivity experiments, PHA blasts were infected with approximately 100 TCID of either the AZT-sensitive strain IIIb or the AZT-resistant strain 691 in the presence or absence of various concentrations of AZT. Cells and supernatant were collected at various times and later analyzed for HIV nucleic acids and p24 antigen, respectively. Figure 1 shows the results of p24 and gene amplification measurements to assess the inhibition of replication

by AZT. DNA and p24 measurements demonstrate that the replication of HIV strain IIIb was significantly inhibited by 0.01–0.1 μM AZT, but greater amounts of AZT were required to inhibit RNA accumulation significantly. HIV strain 691 was significantly less inhibited by AZT, requiring concentrations of at least 0.1 μM AZT to slow replication as measured by p24 antigen. Accumulation of HIV RNA and DNA were not significantly inhibited by as much as 1.0 μM AZT.

Assessment of Viral Replication and AZT Sensitivity in HIV Isolated from HIV-Seropositive Individuals

Virus stocks were prepared from the PBMC of HIV-seropositive individuals, titered, and used to infect PHA blasts in the presence or absence of AZT. HIV *gag* RNA, DNA, and p24 antigen production in these cultures were evaluated as previously described for HIV strains IIIb and 691. Figure 2 shows the p24, RNA, and DNA assay results from cultures of PHA blasts infected with 100 and 50 TCID of virus isolated from patient L2 or patient JR, respectively. Patient L2 had been diagnosed with the acquired immunodeficiency syndrome (AIDS) and had been on AZT therapy for more than 2 years when the virus stock was generated. This virus replicated even in the presence of 1 μM AZT, indicating a relatively high level of resistance to AZT. Patient JR was asymptomatic and had never received AZT treatment. The growth of virus from this patient appeared to be relatively sensitive to AZT, as it was completely inhibited by 0.1 μM AZT. For both patients, all three markers of HIV replication produced similar AZT sensitivity results that were related to their clinical history.

DISCUSSION

We investigated the utility of measuring HIV *gag* RNA and DNA to assess the replication of HIV in cell cultures and to evaluate the effect of drugs on viral replication. Using quantitative PCR techniques, we found that the increases in HIV *gag* RNA and DNA in PHA blasts infected with HIV paralleled the production of HIV p24 antigen in culture supernatants in our short-term assay system. The use of these markers to measure replication of HIV at a rela-

tively low multiplicity of infection (10–100 TCID) within a short time frame (≤ 7 days) demonstrated the replicative capacity of the virus in control cultures and the overall effect of antiretroviral drugs.

While the relationship between the p24, RNA, and DNA measurements was similar in each instance, the change in HIV *gag* RNA expression in the cells preceded and predicted the ultimate changes in p24 antigen and HIV DNA levels within 1–3 days. These results were not unexpected, because the production of structural and regulatory RNA species precedes the production of structural proteins.⁽²⁰⁾ The ineffectiveness of lower AZT concentrations on the replication of both AZT-sensitive and -resistant strains was also evident earlier by RNA analysis. The use of HIV RNA to assess infectivity and antiviral activity may be ultimately more sensitive and allow conclusions to be made days earlier than p24 or DNA measurements.

We used titered virus stocks that had been passaged once through PHA blasts to assess the utility of our assay system. In assessing the effect of AZT on the patient-derived virus, we were limited to a lower TCID when testing one strain (JR) because a single-pass stock that possessed a high virus titer could not be generated. Despite the very low multiplicity of infection used to infect these cultures, we were still able to quantitate viral replication within 3–7 days. The sensitivity of PCR systems might allow relatively small quantities of virus in primary specimens (e.g., plasma, serum, CSF, etc.) to be detected within a short time. A more rigorous analysis of the effect of a varying inoculum size and of different inherent virus growth rates on the drug sensitivity measurements in this type of in vitro system would be needed to further this application of PCR techniques.

Using measurements of HIV nucleic acids to assess viral replication may also be useful in examining the mechanism of action of antiretroviral drugs. Although not evident in our studies using AZT, in some instances the release of p24 antigen into culture supernatants can continue to occur even though HIV mRNA transcription⁽³⁾ and the production of in-

fectious virus particles^(3,4) is inhibited. In addition, the integration of HIV DNA in the absence of productive viral replication has been shown to occur,⁽³⁾ an event that could be detected by

nucleic acid evaluation but not by measuring the production of structural proteins. The sole use of structural proteins such as p24 antigen to assess viral replication could thus obscure the

effectiveness of an antiviral drug that primarily affects the transcription of certain types of viral RNA or DNA.

Our results suggest that gene amplification techniques may be effectively

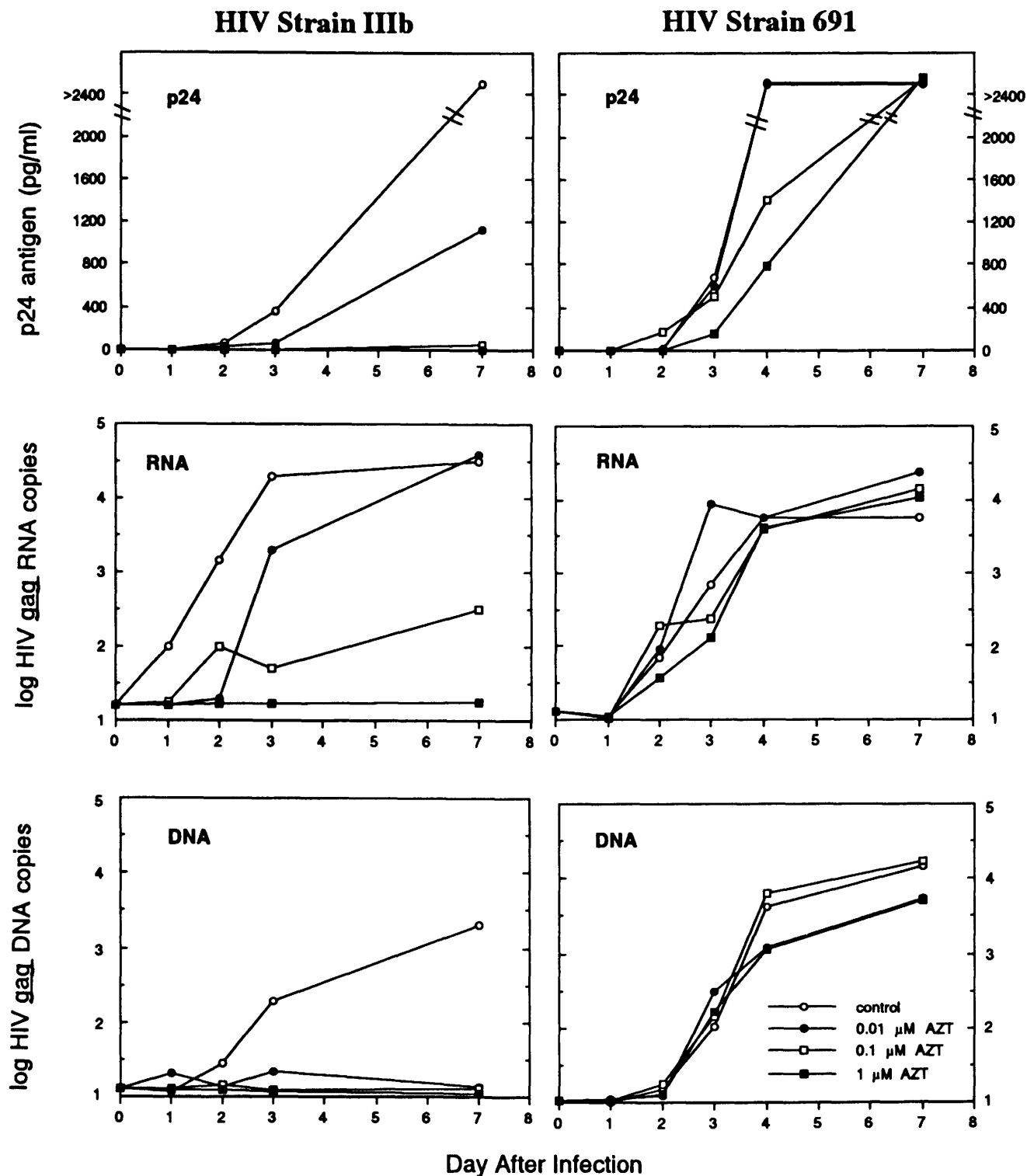


FIGURE 1 Replication of 100 TCID of HIV strain IIIb and strain 691 in PHA blasts. PHA-stimulated normal PBMC were infected with HIV strain IIIb or strain 691 in the presence of various concentrations of AZT. Cells and supernatant were sampled from each culture and assayed for p24 antigen, HIV *gag* RNA, and HIV *gag* DNA. Each point represents a single p24 determination or a single amplification of 1 μ g of cellular DNA or reverse-transcribed cellular RNA.

applied to an in vitro assay system to measure viral replication and antiviral activity. Further assessment of the effect of multiplicity of infection, inherent viral growth rate, host cell characteristics, and interest variability will

be required to develop this type of assay system into a quantitative measure of drug susceptibility. In addition to measuring directly the ability of a drug to inhibit viral replication, RNA and DNA quantitation can also provide

preliminary information regarding mechanism of drug action. Although the current cost (labor and materials) of performing PCR evaluations of RNA and DNA samples is greater than performing p24 antigen measurements,

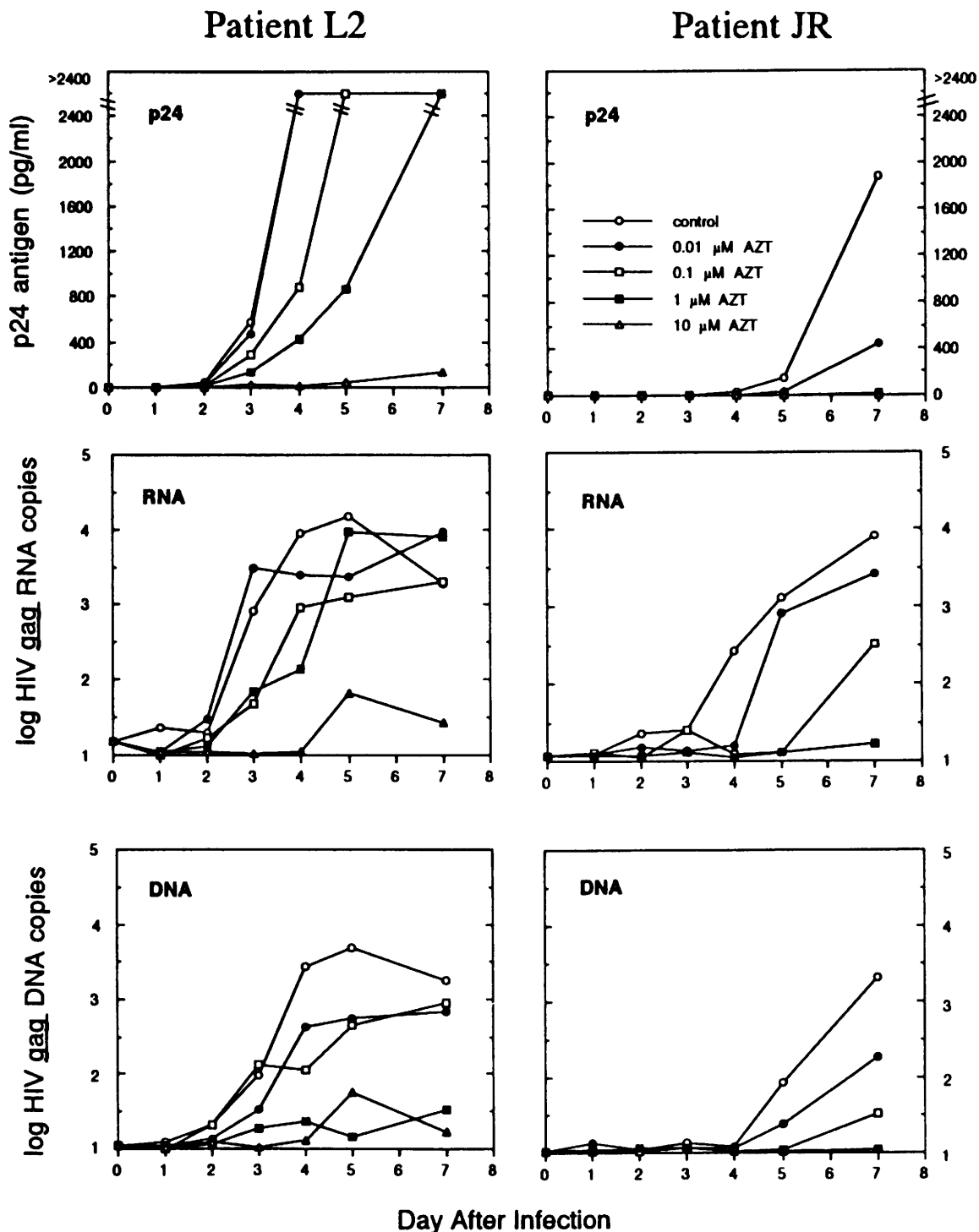


FIGURE 2 Replication of patient-derived HIV in PHA blasts. PHA-stimulated normal PBMC were infected with 100 TCID of patient isolate L2 or 50 TCID of HIV patient isolate JR in the presence various concentrations of AZT. Cells and supernatant were sampled from each culture and assayed for p24 antigen, HIV *gag* RNA, and HIV *gag* DNA. Each point represents a single p24 determination or a single amplification of 1 μg of cellular DNA or reverse-transcribed cellular RNA.

the development of automated equipment and techniques⁽¹⁵⁾ may provide simple and relatively inexpensive means to evaluate HIV replication by quantitating nucleic acids.

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