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Susceptibility testing by polymerase chain reaction DNA quantitation: A method to measure drug resistance of human immunodeficiency virus type 1 isolates

(zidovudine/didanosine/antiviral drugs/reverse transcriptase/clinical trials)

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ABSTRACT **Polymerase chain reaction (PCR) DNA quantitation (PDQ) susceptibility testing rapidly and directly measures nucleoside sensitivity of human immunodeficiency virus type 1 (HIV-1) isolates. PCR is used to quantitate the amount of HIV-1 DNA synthesized after *in vitro* infection of peripheral blood mononuclear cells. The relative amounts of HIV-1 DNA in cell lysates from cultures maintained at different drug concentrations reflect drug inhibition of virus replication. The results of PDQ susceptibility testing of 2- or 3-day cultures are supported by assays measuring HIV-1 p24 antigen production in supernatants of 7- or 10-day cultures. DNA sequence analyses to identify mutations in the reverse transcriptase gene that cause resistance to 3′-azido-3′-deoxythymidine also support the PDQ results. With the PDQ method, both infectivity titration and susceptibility testing can be performed on supernatants from primary cultures of peripheral blood mononuclear cells. PDQ susceptibility testing should facilitate epidemiologic studies of the clinical significance of drug-resistant HIV-1 isolates.**

Human immunodeficiency virus type 1 (HIV-1) isolates resistant to zidovudine (3′-azido-3′-deoxythymidine, AZT) and didanosine (2′,3′-dideoxyinosine, ddI) have been reported, but the role of drug-resistant HIV-1 in pathogenesis is not well defined (1–3). Rapid, quantitative drug susceptibility testing of all isolates, and not only the minority that replicate in MT-2 cells (1, 4), is necessary for epidemiologic evaluation of the contribution of resistant HIV-1 to disease progression. To obtain an HIV-1 isolate from virtually every subject, each HIV-1-infected subject's peripheral blood mononuclear cells (PBMCs) must be cocultivated with an uninfected donor's phytohemagglutinin (PHA)-stimulated PBMCs. We have developed a rapid method for drug susceptibility testing that allows the use of the supernatants of such primary cocultures. The polymerase chain reaction (PCR) is used to determine relative amounts of HIV-1 DNA in cultures maintained in different drug concentrations following *in vitro* infection. The results of this PCR DNA quantitation (PDQ) susceptibility testing are supported by HIV-1 p24 antigen-based susceptibility assays (5) and by DNA sequencing of codons in the HIV-1 reverse transcriptase (RT) gene in which specific mutations cause AZT resistance (2).

MATERIALS AND METHODS

Cell and Virus Culture. Primary PBMC coculture supernatants were used. These were in excess of those stored for use in virologic assessment of clinical protocols. Primary coculture of HIV-1 was performed with 5×10^6 3-day PHA (10 μg/ml; Difco)-stimulated PBMCs from a single HIV-1-seronegative blood donor and 10×10^6 PBMCs (or 1 ml of plasma) from an HIV-1-infected subject (5). Virus stocks were generated from passage of primary coculture supernatants on PHA-stimulated PBMCs (5).

Prior to each PDQ experiment, virus inocula were first filtered (Millex HV, 0.45 μm, Millipore) and then treated with RQ1 RNase-free DNase (Promega) in 1 mM $MgCl_2$ (4 units of DNase per ml of culture supernatant) for 20 min at room temperature. Heat-inactivated (70°C, 30 min) virus inoculum served as a control for the adequacy of filtering and DNase treatment in eliminating input HIV-1 DNA (6).

PDQ HIV-1 Infectivity Titration. Aliquots (150 μl) of serial dilutions of virus sample were used to infect 2×10^6 PHA-stimulated donor PBMCs in 1.5 ml of growth medium (5) per well of a flat-bottom 24-well plate (Corning). Separate cell samples were counted, harvested, and lysed at 48, 72, and 96 hr. Quantitative PCR and HIV-1 copy-number determination were then performed in duplicate on each lysate.

PDQ Susceptibility Testing. The results of a PDQ infectivity titration assay were used to determine the virus dilution and length of culture time employed in a subsequent PDQ susceptibility test. These parameters were chosen so that the yield of HIV-1-specific PCR product for the untreated control infection would fall on the HIV-1 copy-number standard curve before the curve approached its asymptotic maximum, or plateau. PHA-stimulated donor PBMCs were incubated with drug for 4 hr prior to infection. Duplicate wells in a 24-well plate received identical HIV-1 inocula for each drug concentration tested and for the untreated infected controls. Uninfected controls and drug toxicity controls were included in each experiment. All cultures were harvested and cells were lysed for PCR after either 48 or 72 hr. The entire experiment was repeated without analysis whenever HIV-1 DNA was amplifiable from input HIV-1 DNA controls (i.e., heat-inactivated virus inoculum). Previously characterized isolates were used as assay standards in each experiment (1, 5).

HIV-1 p24 Antigen-Based Susceptibility Testing. Susceptibility testing based on enzyme-linked immunoassay (DuPont/NEN) of HIV-1 p24 antigen was performed with 7- or 10-day cultures (5). Virus stocks were used in this assay following infectivity titration by end-point dilution (7).

Quantitative PCR. Cell pellets were lysed in various volumes of lysis buffer (50 mM KCl/10 mM Tris·HCl, pH 8.3/2.5 mM $MgCl_2$/0.5% Nonidet P-40/0.5% Tween 20/0.01% proteinase K) to yield a concentration of 1.2×10^4 cell equivalents/μl (8). Uniformity of cell lysate DNA concentrations was confirmed in representative experiments by enhance-

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Abbreviations: PBMC, peripheral blood mononuclear cell; PDQ, PCR DNA quantitation; RT, reverse transcriptase; HIV-1, human immunodeficiency virus type 1; AZT, 3′-azido-3′-deoxythymidine; ddI, 2′,3′-dideoxyinosine; ELOSA, enzyme-linked oligonucleotide solution sandwich hybridization assay; PHA, phytohemagglutinin.

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ment of Hoechst 33258 fluorescence (9) (Mini-Fluorometer, Hoefer).

A conserved primer pair was synthesized according to the HIV-1 LAI (formerly BRU) sequence, GenBank accession no. K02013: 5′ primer (sense strand), bases 1889–1908; 3′ primer (antisense strand), bases 2211–2192. The primers were used to amplify a 323-base-pair fragment of the HIV-1 *pol* gene from 1.2×10^5 cell equivalents of lysate by using PCR (GeneAmp, Cetus) under previously described conditions (10). Following one cycle of 2 min at 94°C, 1 min at 45°C, and 1 min at 72°C, an additional 29 cycles were performed with 1 min at each of those temperatures. Amplifications were repeated if HIV-1 DNA was amplifiable from reagent controls.

HIV-1 *pol* gene amplification products were specifically detected and quantitated as described (11). Heat-denatured PCR products were hybridized in a streptavidin-coated microtiter plate well with both biotinylated capture probe and horseradish peroxidase (HRP)-labeled detector probe [enzyme-linked oligonucleotide solution sandwich hybridization assay (ELOSA), DuPont Medical Products, Billerica, MA] for 60 min at 37°C. After extensive washing to remove all reactants except probe–DNA hybrids, an HRP chromogen, tetramethylbenzidine (TMBlue, Transgenic Sciences, Worcester, MA), was added to each well. The HRP-catalyzed color development was stopped after 1 hr by addition of sulfuric acid to 0.65 M. Absorbance (OD) at 450 nm was measured in an automated microtiter plate reader (SLT Labinstruments, Hillsborough, NC).

A standard curve of HIV-1 DNA copy number was generated in each PCR by using a dilution series of ACH-2 cells (National Institutes of Health AIDS Research and Reference Reagent Program, Rockville, MD) containing one proviral HIV genome per cell (12). Prior to lysis, 4-fold serial dilutions of ACH-2 cells were made with HIV-1-seronegative, PHA-stimulated PBMCs to a final concentration of 1.2×10^4 cells per μl.

Data Analysis. The standard curve for HIV DNA copy number was based on a modified log-logit equation: $OD = a + [(c - a)/\{1 + \exp[b - d(\ln \text{copy number})]\}]$. The constant a represents background signal from a negative control, defined as twice the OD of a reagent control. The other constants were determined by computerized estimation (Systat version 5.1, Systat, Evanston, IL; nonlinear regression modeling, default options) from the ACH-2 cell dilution series results in each amplification. The ACH-2 standard curves each had a corrected $r^2 \geq 0.94$, and in 85% of the experiments the r^2 was ≥ 0.99.

The 50% inhibitory concentration (IC_{50}) was determined from either PDQ or p24 data by computerized estimation (Systat, as above) of the median-effect equation: fractional reduction = $1/[1 + (IC_{50}/\text{drug concentration})^m]$ (13). The corrected r^2 for each PDQ IC_{50} determination was ≥ 0.86, except for one value that was 0.67.

DNA Sequence Analysis. A 545-base-pair fragment of the HIV-1 RT gene was PCR amplified from cell lysates by use of primers from the HIV LAI sequence: 5′ primer (sense strand), bases 2284–2307; 3′ primer (antisense strand), bases 2829–2809. Gel-purified PCR products were directly sequenced (5).

RESULTS

PDQ measures the amount of HIV-1 DNA synthesized after *in vitro* infection relative to a standard curve of HIV-1 DNA copy number. A curve is constructed in each experiment by amplification of known numbers of ACH-2 cells diluted with HIV-1-seronegative donor PBMCs. Amplification results, under identical reagent and thermal cycling conditions, of five replicates of each point on such a standard curve are depicted in Fig. 1*A*. The 95% confidence intervals (twice the standard deviation) about the mean amplification yields do not overlap in the portion of the curve between 16 and 1024 copies (Fig. 1*A*). The PCR yield plateaus above 1024 copies of preamplification template.

An initial titration of the infectivity of a virus sample was done by PDQ to determine conditions necessary for comparison of relative amounts of HIV-1 DNA synthesized in infected cultures at different drug concentrations. To ensure adequate quantitation of drug effect, the untreated control infection copy number must fall on the pre-plateau portion of the standard curve. An example of a typical PDQ susceptibility test using a primary PBMC coculture supernatant is depicted in Fig. 1*B*. Results of duplicate amplifications of each of two infections at each drug concentration clustered closely on the standard curve (Fig. 1*B*), thereby allowing quantitation of the fractional reduction of mean copy number versus AZT concentration (Fig. 2*A*). Representative median-effect plots testing previously characterized isolates (1, 5) are depicted in Fig. 2.

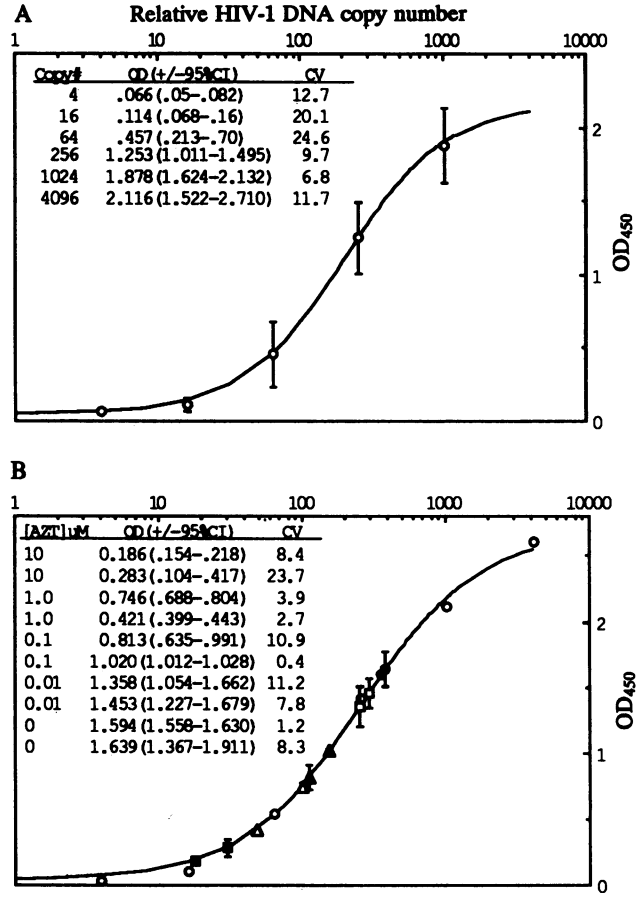

FIG. 1. Standard curves of relative HIV-1 DNA copy number per 1.2×10^5 cell equivalents were constructed by amplification of a dilution series of ACH-2 cells; each ACH-2 cell contains a single proviral genome (12). The PCR product yields were measured by OD at 450 nm following the ELOSA assay (see *Materials and Methods*). (*A*) Five replicates of each sample of the ACH-2 cell dilution series (○) are depicted. Error bars indicate twice the standard deviation above and below the mean [95% confidence interval (CI)]. The actual data, as well as the coefficient of variation (CV) of replicate amplifications, are shown in the *Inset*. (*B*) An example of a PDQ susceptibility experiment which includes: the standard curve (○), the mean amplification yield of duplicate amplifications (with error bars indicating 95% CIs) of duplicate infections in the absence of AZT (●) or in the presence of 0.01 μM AZT (□), 0.1 μM AZT (▲), 1.0 μM AZT (△), or 10 μM AZT (■). The virus inoculum was a primary PBMC coculture supernatant (isolate 349d-3/91). The absolute data and the CV of each duplicate amplification are shown in the *Inset*.

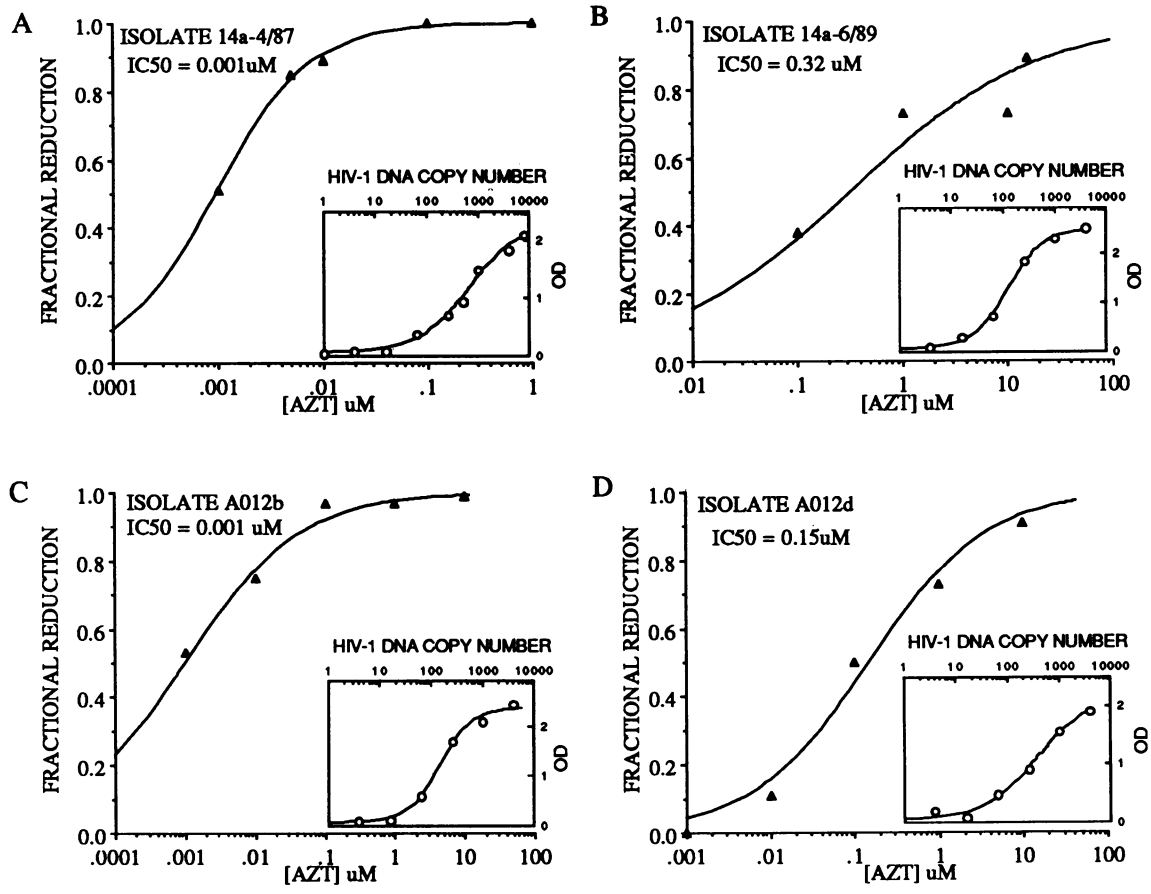

FIG. 2. Median-effect plots from AZT PDQ susceptibility tests. Data for two pairs of clinical isolates previously characterized as AZT-sensitive [A012b (1), 14a-4/87 (5)] or AZT-resistant [A012d (1), 14a-6/89 (5)] are shown. Fractional reduction (▲) equals 1 minus the relative HIV-1 DNA copy number obtained at each drug concentration divided by the relative HIV-1 DNA copy number of untreated infected controls. Relative HIV-1 DNA copy numbers were calculated using ACH-2 standard curves (*Insets*) from coamplification of a 4-fold dilution series of ACH-2 cells.

The AZT IC_{50} values determined by PDQ susceptibility testing were supported by two independent methods: HIV-1 p24 antigen-based susceptibility testing using 7- or 10-day cultures and DNA sequence analysis of four codons of the RT gene implicated in AZT resistance (2) (Table 1). Isolates obtained from subjects with minimal or no AZT exposure [14a-4/87 (5), A012b (1), 45P-6/90] had only wild-type codons dominating at these positions and PDQ IC_{50} values ≤ 0.003 μM (Table 1 and Fig. 2 *A* and *C*). Two isolates (349d-3/91, 27-4/89), each obtained after 6–9 months of AZT therapy, demonstrated a 17- to 60-fold increase in PDQ IC_{50} relative to the fully susceptible isolates and had only one of the mutant RT codons (Table 1 and Fig. 3 *A* and *C*). Isolates obtained after 12–24 months of AZT therapy [14a-6/89 (5), A012d (1), 50-7/89, 100h-3/91] each contained at least the two mutated RT codons that cause high-level resistance (2), codons 70 and 215 (Table 1). The IC_{50} values in the PDQ assay were ≥ 0.15 μM for these isolates, 50- to 320-fold greater than for the fully susceptible isolates (Table 1, Fig. 2 *B* and *D*, Fig. 3 *B* and *D*). IC_{50} values as determined by PDQ or p24 antigen assays were similar to one another for AZT (Table 1) and for ddI (Table 2). In both assays, the ddI IC_{50} values were within a narrow range for isolates from ddI-naive subjects (Table 2).

Table 1. Comparison of AZT PDQ IC_{50} values with p24 antigen assay IC_{50} values and RT sequence

Isolate	Mo.*	IC_{50}, μM		Mutant RT codons†
		PDQ	p24	
14a-4/87	0	0.001	0.004	None
A012b	2	0.001	0.006	None
45P-6/90	4	0.003	NT	None
27-4/89	6	0.06‡	0.024	70
		0.06	0.015	
349d-3/91	9	0.05‡	NT	215
100h-3/91	19	0.2‡	NT	70, 215
14a-6/89	24	0.32	0.64	67, 70, 215, 219
A012d	24	0.15	0.287	67, 70, 215, 219
50-7/89	22	0.19	0.4	67, 70, 215, 219

NT, not tested.
*Months on continuous AZT therapy.
†Indicates which of RT codons 67, 70, 215, and 219 (2) have a dominant mutant genotype in the isolate.
‡Primary PBMC coculture supernatant was inoculum. Virus stocks were inocula in other experiments.

PDQ was used to titrate the infectivity of supernatant fluids from 10 unselected HIV-1-positive primary PBMC cocultures to determine the feasibility of directly studying such fluids. PBMC lysates were found to contain an amount of HIV-1 DNA adequate for copy-number comparisons within 72 hr of *in vitro* infection with 9 of these samples. The 10th supernatant fluid was from a coculture that became HIV-1 p24-positive only after 26 days and was therefore likely to have a low titer of virus. A lysate of PBMCs infected for 72 hr with this sample required 20 more cycles for positivity.

Two of these primary coculture supernatants (349d-3/91 and 100h-3/91), as well as two others, were studied by PDQ susceptibility testing (Fig. 3). The PDQ IC_{50} values were consistent with RT gene sequencing results (Table 1). However, the IC_{50} values of primary coculture supernatants could

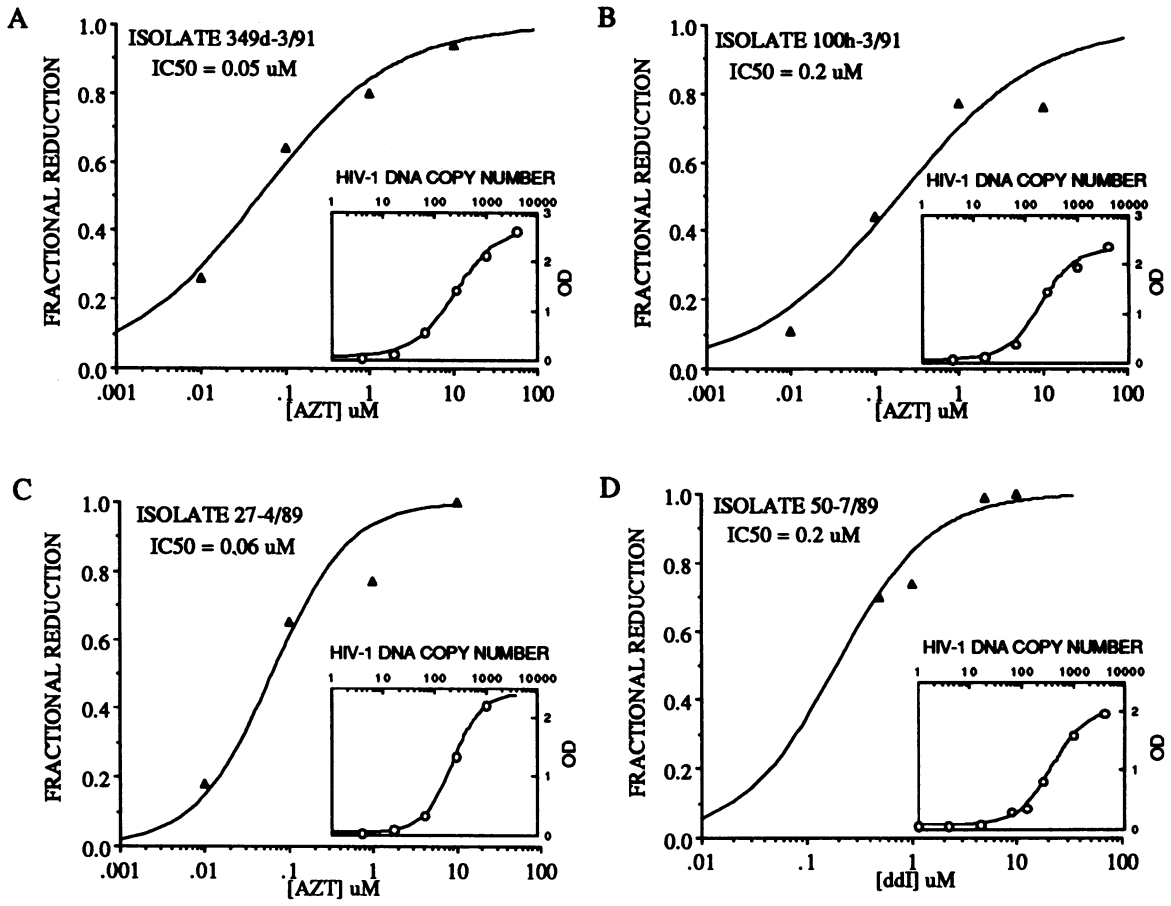

FIG. 3. Median-effect plots from PDQ susceptibility tests (three AZT and one ddI) using supernatants from primary PBMC coculture of four clinical isolates. The ACH-2 cell standard curve for each experiment is shown (*Insets*).

not be confirmed by the previously described p24 susceptibility assay because a larger amount of virus inoculum was needed for that method (5). We did compare the results of both PDQ and p24 testing of passaged, higher-titer virus to those of PDQ testing of primary coculture supernatants. The IC_{50} values for these passaged viruses were similar to those of the coculture supernatant viruses whether determined by PDQ (isolate 27-4/89, Table 1) or the p24 assay (isolate 27-4/89, Table 1; isolate 50-7/89, Table 2).

Table 2. Comparison of ddI PDQ IC_{50} values with p24 antigen assay IC_{50} values

Isolate	IC_{50}, μM	
	PDQ	p24
14a-4/87	0.83*	0.34
	0.55†	0.49
A012b	0.3	0.38
14a-6/89	0.36	0.29
A012d	0.63*	0.64
	0.85†	0.88
50-7/89	0.2‡	0.46
17-3/89	0.55	0.24
1-10/88	0.75	0.46

All isolates were from subjects who never received ddI.
*Because initial infections yielded too high a copy number when undiluted cell lysates were amplified, infections were repeated with a lower multiplicity of infection and the resulting cell lysates were amplified without dilution.
†Because initial infections yielded too high a copy number when undiluted cell lysates were amplified, dilutions of the cell lysates were amplified.
‡Primary PBMC coculture supernatant was inoculum. Other experiments used virus stocks as inocula.

DISCUSSION

PDQ susceptibility testing has been validated for the isolates studied by comparison to an HIV-1 p24 antigen-based assay and by DNA sequencing analyses of RT gene mutations (Tables 1 and 2). The PDQ method offers significant advantages over other antiretroviral-drug resistance assays. Small volumes of low-titer virus-containing supernatants from primary PBMC cocultures, rather than passaged virus, can be used to test drug sensitivity, thus minimizing the selection bias of *in vitro* culture (14). Occasional selection of subpopulations of HIV-1 with passage has been seen (15). Although p24-antigen based assays can be used on primary PBMC coculture supernatants, PCR sensitivity should allow PDQ testing of isolates with very low titers. Isolates with too low a titer for testing by the 30-cycle amplification described here may still be testable by PDQ using a greater number of PCR cycles and a lower range of copy-number standards. However, the susceptibility of the one virus that required a 50-cycle PDQ infectivity titration to yield signal was not tested here.

The flexibility of PCR may also obviate the need for infectivity titration if the IC_{50} values of all isolates are determined at similar levels of amplification yields, within the range of the standards that permits quantitation. If product yield from untreated control cells infected with a high multiplicity of virus falls on the plateau portion of the standard curve when amplified undiluted, then a dilution of the cell lysates can be amplified to allow copy-number comparisons at different drug concentrations. In testing two isolates for resistance to ddI (14a-4/87 and A012d), the IC_{50} values calculated from amplifications of a dilution of cell lysates from an initial high-multiplicity infection were similar to

those determined from a repeated, lower-multiplicity infection (Table 2). In other experiments (data not shown), decreasing the number of PCR cycles from 30 to 26 also decreased the amplification yield from an initial plateau-level copy number to within the range that allows comparisons.

PDQ has allowed quantification of the degree of resistance of all isolates tested, including highly AZT-resistant isolates. Such highly AZT-resistant isolates have been reported to have IC_{50} values greater than the highest drug concentration tested in other PBMC-based assays (4, 16). Two theoretical advantages to PDQ susceptibility testing may explain this difference. One advantage may result from the short duration of time needed in culture. AZT-sensitive HIV-1 can be isolated from an *in vitro* culture after an extended period, despite the presence of AZT (17). After extended culture, virus isolates with differences in both replication rate and drug susceptibility may appear to have the same IC_{50} values. The early culture end point for PDQ may minimize the risk of artifactually increased IC_{50} values for rapidly replicating viruses that could result from this phenomenon. A second theoretical advantage of PDQ testing is specific to agents that inhibit reverse transcription. It is possible that susceptibility tests measuring HIV-1 p24 antigen or RNA production in cultures treated with an RT inhibitor may be less able than PDQ to distinguish a few infected cells making a large amount of these virus products from many infected cells. PDQ may more directly reflect virus spread in such cultures because it directly measures the reverse-transcribed DNA. The heat-inactivated virus controls ensure that only newly synthesized HIV-1 DNA is detected in the PDQ tests. Resistance of input HIV-1 DNA to DNase has not been noted.

PDQ susceptibility testing has not yet been limited by the genetic diversity of HIV-1 isolates, although theoretically it is possible that some isolates may not be amplifiable with the primers used here. This single, highly conserved primer pair has amplified HIV-1 DNA from all clinical isolates studied here, as well as from 57 of 59 HIV-1-seropositive individuals' PBMCs in an earlier study (10). The ability to rapidly test HIV-1 isolates from virtually every subject, with minimal selection pressure from *in vitro* culture, as well as the theoretical possibility of fewer artifacts than with other methods, suggests that PDQ will be useful for epidemiologic investigation of the pathogenic role of drug-resistant HIV-1.

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