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Detection of human immunodeficiency virus type 1 clinical isolates with reduced sensitivity to zidovudine and dideoxyinosine by RNA·RNA hybridization

(AIDS/resistance)

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ABSTRACT **A quantitative rapid assay to detect resistant clinical human immunodeficiency virus type 1 (HIV-1) strains remains an important medical goal. A system incorporating a quantitative RNA·RNA hybridization assay that measures the amount of intracellular HIV-1-specific RNA has been employed to detect the level of inhibition by nucleoside analogues in sensitive and resistant HIV-1 strains. The RNA·RNA hybridization assay readily distinguished previously published zidovudine (ZDV; 3′-azido-3′-deoxythymidine)-resistant isolates from ZDV-sensitive isolates of HIV-1. The 50% inhibitory concentration (IC_{50}) of ZDV for $HTLV\text{-}III_B$ and sensitive clinical HIV-1 isolates is between 0.01 and 0.04 μM. HIV-1 strains from three patients on long-term ZDV therapy displayed a greater than 20-fold increase in the ZDV IC_{50} compared to sensitive strains. The drug sensitivity system was confirmed by showing that mutations in the HIV reverse transcriptase gene from a ZDV-resistant isolate resulted in four amino acid changes (Leu-125 → Trp, Ile-142 → Val, Thr-215 → Tyr, and Pro-294 → Thr) including one change (Thr-215 → Tyr) that has been previously reported to be associated with resistance. One clinical HIV strain with high-level ZDV resistance displayed a 5-fold increase in 2′,3′-dideoxyinosine IC_{50} compared to that of $HTLV\text{-}III_B$. A drug sensitivity assay employing RNA·RNA hybridization may be useful for extensive screening of HIV isolates from patients enrolled in clinical trials and permit the correlation of *in vitro* resistance with clinical outcome.**

The human immunodeficiency virus type 1 (HIV-1) has extensive *in vivo* genotypic variation (1). In single-cycle replication experiments, a model retrovirus, Rous sarcoma virus, has been shown to have a very high mutation rate, indicating that a high percentage of replicating retroviruses is likely to contain a large number of mutations (2). Within an individual patient, multiple similar, yet distinct, viral strains that have varying tropism for human lymphocytes, monocytes, and other cells can be isolated (3). Although the use of zidovudine [ZDV; 3′-azido-3′-deoxythymidine (AZT)] has been shown to extend life in patients with AIDS and delay the progression to AIDS in some asymptomatic HIV-1-positive adults, the efficacy of ZDV is short lived, and clinical deterioration ensues (4–7). The clinical significance of chronic ZDV therapy on *in vivo* selection for HIV strains that are resistant to antiviral agents or to more highly pathogenic strains remains to be determined, but with other viruses, such as herpes simplex, cytomegalovirus, and influenza, resistant viral strains are clinically important (8–10). Unfortunately, no objective clinical manifestations or surrogate laboratory markers predict the emergence of clinically relevant resistant HIV isolates. There is a need to develop an *in vitro* assay that can reliably detect resistant HIV-1 strains. A system using HeLa HT46C cells in a plaque reduction assay to measure drug sensitivity of clinical isolates has been shown to detect resistant HIV-1 strains (11). The 50% inhibitory concentration (IC_{50}) of isolates from patients without prior ZDV therapy ranged between 0.01 μM and 0.05 μM. In patients who received more than 6 months of ZDV, the HIV isolates exhibited reduced sensitivity to ZDV, with IC_{50} values between 0.06 μM and 4.0 μM (12). Unfortunately, coculturing patients' peripheral blood mononuclear cells (PBMCs) with a continuous cell line resulted in a viral isolation rate of only 30%. While the mechanism for ZDV resistance is unknown, mutations in the HIV-1 reverse transcriptase (RT) may decrease binding to ZDV triphosphate, rendering ZDV less able to inhibit chain elongation in viral reverse transcription. Nucleotide sequence analysis of the HIV-1 RT coding region in five sensitive and resistant pairs showed three amino acid substitutions (Asp-67 → Asn, Lys-70 → Arg, and Thr-215 → Phe or Tyr) in all of the resistant isolates and a fourth (Lys-219 → Gln) in three of the resistant isolates (13). Although such findings are provocative, only a small number of isolates were examined. A major limitation in a broad application of the HeLa HT46C system is that it can be used to evaluate only syncitial-forming strains of HIV-1.

This paper describes an RNA·RNA hybridization assay that has been applied to detect sensitive and resistant strains of HIV-1 from patients. The assay can be employed to study all isolates that are able to be cultured in PBMCs. By measuring the amount of HIV-1 RNA produced in the acute HIV infection of PBMCs in tissue culture, this assay may provide an objective quantifiable method to establish the clinical significance of HIV resistance and to evaluate cross-resistance to other antiviral drugs. Moreover, this assay may guide the development of combination therapy for patients at all stages of HIV infection.

MATERIALS AND METHODS

Subjects. Participants in clinical protocols sponsored by the National Institutes of Health (NIH) AIDS Clinical Trials Group at Harvard/Boston City Hospital (Beth Israel Hospital Unit, Boston) were invited to enroll in a longitudinal program studying the clinical significance of HIV resistance to antiviral agents. After informed consent was obtained, HIV cultures were performed.

HIV Culture. The Beth Israel Hospital Virology Laboratory is an NIH AIDS Clinical Trials Group certified laboratory for HIV culture and HIV p24 antigen detection. The

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Abbreviations: ddI, 2′,3′-dideoxyinosine; HIV-1, human immunodeficiency virus type 1; PBMCs, peripheral blood mononuclear cells; RT, reverse transcriptase; ZDV, 3′-azido-3′-deoxythymidine (AZT); PHA, phytohemagglutinin.

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method for HIV culture follows the AIDS Clinical Trials Group virology reference laboratory consensus culture protocol (14). PBMCs from HIV-negative donors were isolated by Ficoll-Paque (Pharmacia) and phytohemagglutinin (PHA; 3 μg/ml; PHA-P, Sigma) stimulated for 72 hr. PBMCs from study subjects were isolated by Ficoll-Paque and cocultured with washed PHA-stimulated donor cells in a ratio of 1:1. Fresh PHA-stimulated cells were added weekly, and one-half volume of coculture medium was replaced every 3 or 4 days. Supernatant was assayed for p24 (Abbott) twice weekly. Supernatants from all positive cultures were stored at −70°C.

Control Viral Isolates. Wild-type HIV-1 was obtained from the supernatant of H9 cells infected with HTLV-III_B. Two reference sensitive/resistant pairs of HIV-1 isolates [G762-3/G691-2 and H112-2/G910-6, previously published as AO12 pre/post ZDV and AO18 pre/post ZDV (12), respectively] were obtained from the NIH AIDS Research and Reference Reagent Program.

Drugs. ZDV was generously provided by Burroughs Wellcome, and 2',3'-dideoxyinosine (ddI) was purchased from Calbiochem.

Drug Sensitivity Studies. PBMCs from HIV-negative donors were isolated from Ficoll-Paque (Pharmacia) and were PHA stimulated for 72 hr. After PHA stimulation, PBMCs were washed, counted, and infected for 2 hr with supernatant from clinical and laboratory HIV cultures at a multiplicity of infection of 0.001 pg of HIV p24 per cell. PBMCs were then washed twice and divided into culture vessels with medium [RPMI 1640 with glutamine, 20% fetal bovine serum (heat inactivated), 5% purified human interleukin 2 (Cellular Products), penicillin (100 units/ml), and streptomycin (100 μg/ml)] and increasing concentrations of ZDV (0.001, 0.1, 1.0, or 10.0 μM) or ddI (1.0, 5.0, 10.0, or 100.0 μM). On day 7, cells were harvested and counted, and RNA was isolated in 5 M guanidium thiocyanate (1×10^7 cells per ml) and stored at −20°C for later hybridization.

RNA·RNA Hybridization. RNA from 2×10^5 cells was hybridized by target cycling with a mixture of HIV-1 probes: the ^{125}I-labeled pGAP RNA detector probe, specific for the HIV-1 RT gene, and three poly(dA) capture probes consisting of oligonucleotides of regions designed to hybridize to viral RNA nucleotide sequences adjacent to the ^{125}I-labeled pGAP RNA probe (Gene-Trak Systems, Framingham, MA) (15). Viral nucleic acid–target probe complexes were initially captured by oligo(dT) magnetic particles. Nonspecific background hybridization was reduced by washing, eluting, and recapturing the complexes. Hybrids were placed on a nitrocellulose membrane and subjected to autoradiography for 24–48 hr. RNA quantitation was performed by densitometric scanning of the dot blot on x-ray film (LKB model 2222-020 UltroScan XL densitometer; Pharmacia).

IC_{50} Determination. Autoradiographs were scanned by the densitometer, quantified by calculation of the area under the curve, and expressed as a fraction of the control. The inhibition curve for each viral isolate was developed by plotting the area under the curve against antiviral drug concentration by using the exponential regression curve ($Y_i = a\ 10^{(6x_i)}$ + error). r^2 values for sensitive isolates ranged between 0.778 and 0.998. A quality control curve was constructed for each experiment.

Cloning and Nucleotide Sequence Analysis of the RT Gene from EP113089. DNA was extracted from PBMCs in an HIV coculture of PBMCs from isolate EP113089 and a normal donor. The 1.7-kilobase HIV-1 RT DNA fragment was obtained by polymerase chain reaction amplification as described by Larder and Kemp (13). The fragment was digested with *Eco*RI and *Xba* I (Promega) and cloned into a plasmid vector pGem 3B (Promega). Deoxynucleotide sequence analysis was determined by using a Sequenase kit (United States Biochemical).

RESULTS

RNA·RNA hybridization drug sensitivity assays were carried out by using ZDV and ddI for the wild-type HIV-1 (HTLV-III_B). The ZDV IC_{50} for HTLV-III_B was 0.01 μM and the ddI IC_{50} was 2.2 μM (Figs. 1 and 2 and Table 1). The reference sensitive/resistant pairs of HIV isolates obtained from the NIH AIDS Research and Reference Reagent Program (G762-3/G691-2 and H112-2/G910-6) were tested (Figs. 3 and 4 and Table 1). The G762-3 and H112-2 isolates had ZDV IC_{50} values of 0.02 μM and 0.03 μM, respectively; ddI IC_{50} values of these isolates were 2.5 μM and 6.0 μM, respectively. The G691-2 and G910-6 isolates had ZDV IC_{50} values of >10.0 μM and ddI IC_{50} values of 4.0 μM and 10.0 μM, respectively.

Several clinical HIV isolates from the Harvard AIDS Clinical Trials Group (Beth Israel Unit) were tested for sensitivity to ZDV.

Case 1. EP113089 is an isolate from a patient with AIDS who had initially responded to ZDV but after 6 months on ZDV began to note recurrent fevers. The isolate obtained after 1 year on ZDV was assayed by the RNA·RNA hybridization drug sensitivity assay (Fig. 1) and revealed a ZDV IC_{50} of 2.0 μM, a value 200-fold higher than that of the wild-type strain (Table 1). The ddI IC_{50} was 5-fold higher than that of the wild-type strain. This patient, shortly thereafter, developed an opportunistic infection and expired.

The complete nucleotide sequence of the RT gene from EP113089 revealed 18 nucleotide changes resulting in eight amino acid changes. Published sequences for wild-type HIV-1 show conservation in four of the amino acid residues (Arg-125 → Trp, Ile-142 → Val, Thr-215 → Tyr, and Pro-294 → Thr) (Fig. 5) and lack of conservation in four other amino acid changes (Ile-178 → Met, Gln-207 → Glu, Leu-214 → Phe, and Thr-286 → Ala).

Case 2. B101990 is a clinical isolate from a patient with AIDS-related complex since May of 1988 and who had been on ZDV for 2 years prior to enrollment into a double-blind ZDV vs. ddI clinical protocol. At enrollment, the RNA·RNA hybridization drug sensitivity assay showed that the ZDV IC_{50} was 1.0 μM and that the ddI IC_{50} was 4.0 μM (Table 1 and Fig. 2). After 5 months on study, the patient remains stable.

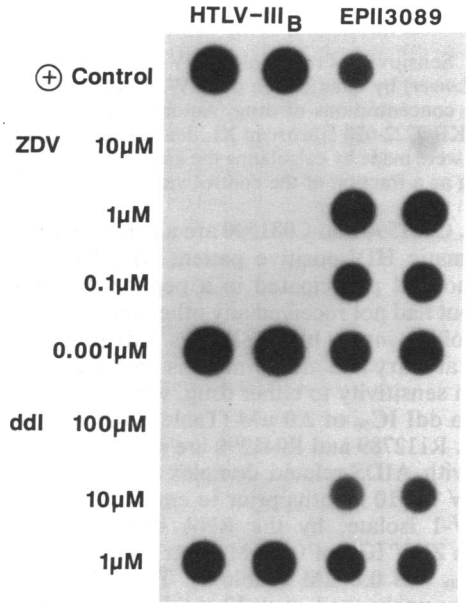

FIG. 1. Twenty-four-hour autoradiograph measuring HIV-1 RNA from 2×10^5 PBMCs following acute infection with HIV strain HTLV-III_B or EP113089 and a 7-day incubation in the presence of various concentrations of ZDV or ddI.

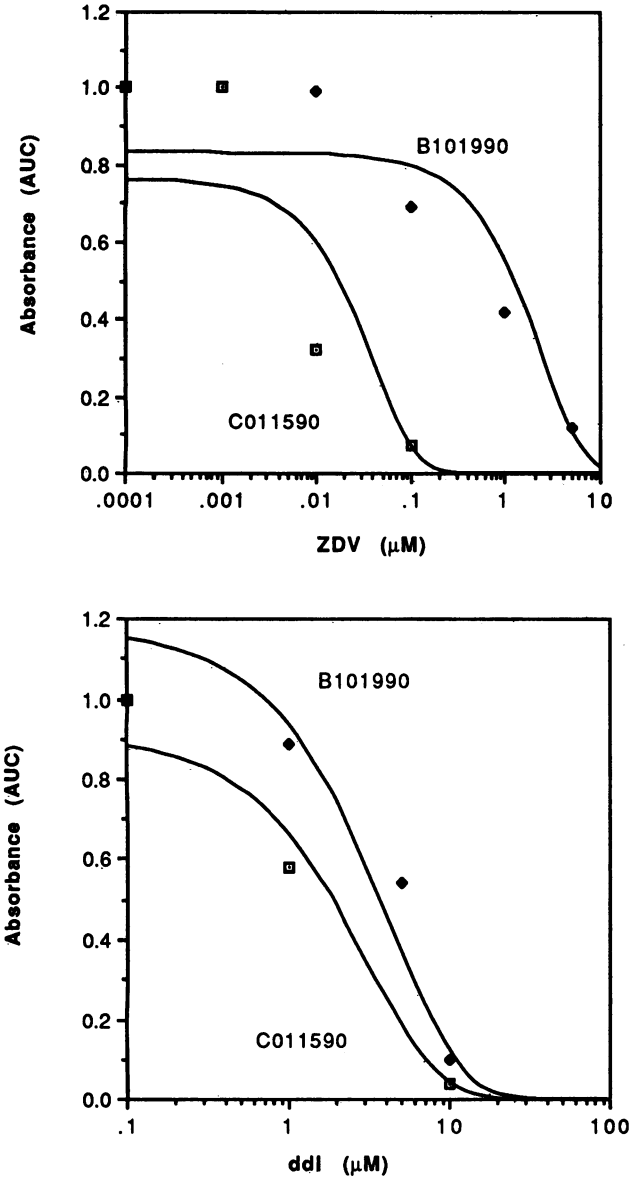

FIG. 2. Sensitivity of two clinical HIV-1 isolates to ZDV (*Upper*) and ddI (*Lower*) by quantitation of HIV-1 RNA in the presence of increasing concentrations of drug. Autoradiographs were scanned with an LKB 2222-020 Ultroscan XL densitometer, and IC_{50} determinations were made by calculating the area under the curve (AUC), expressing as a fraction of the control value.

Case 3. C011590 and C081590 are a pair of isolates from an asymptomatic HIV-positive patient with CD4 <200 for 2 years who had participated in a peptide T protocol for 6 months but had not received any other antiretroviral therapy until enrolling into a blind ZDV vs. ddI protocol. Isolates obtained at entry and after 7 months on protocol showed no change in sensitivity to either drug, with a ZDV IC_{50} of 0.01 μM and a ddI IC_{50} of 2.0 μM (Table 1 and Fig. 2).

Case 4. R112789 and R041290 are a pair of isolates from a patient with AIDS-related complex who had been treated with ZDV for 10 months prior to entry into a ddI protocol. The HIV-1 isolate, by the RNA drug sensitivity assay, showed a ZDV IC_{50} of 0.2 μM; after 4 months off ZDV, the ZDV IC_{50} was 0.6 μM (Table 1). The ddI sensitivity was essentially unchanged over 12 weeks of therapy with ddI. After 8 months, the patient is stable in the study.

Case 5. M011190 and M032690 are a pair of isolates from a patient with AIDS who had been on ZDV therapy for 2 months prior to obtaining the first clinical isolate showing a ZDV IC_{50} of 0.04 μM and a ddI IC_{50} of 2.0 μM (Table 1). The patient entered into a protocol and was randomized to either ZDV or ddI. After 2 months on the study, another clinical isolate was obtained, which showed an increase in the ZDV IC_{50} to 0.11 μM and doubling of ddI IC_{50}. After 8 months on study the patient remains well.

Table 1. Sensitivity of clinical HIV-1 isolates to ZDV and ddI

Isolate	IC_{50} ZDV, μM	IC_{50} ddI, μM	Previous ZDV therapy, mo
H9/HTLV-III$_B$	0.01	2.2	
G762-3	0.02	2.5	2
G691-2	>10.0	4.0	26
H112-2	0.03	6.0	0
G910-6	>10.0	10.0	14
EP113089	2.0	10.0	12
B101990	1.0	4.0	14
C011590	0.02	2.0	0
C081590	0.01	2.0	7*
R112789	0.20	2.5	10
R041290	0.60	3.5	†
M011190	0.04	2.0	0
M032690	0.11	4.0	2*

*The patient was enrolled in a blind trial and received either ZDV or ddI for 7 months or 2 months as indicated.
†The patient was not receiving ZDV for 4 months but was receiving ddI.

DISCUSSION

The quantitative analysis of HIV-1 RNA transcripts has been shown to be useful as a drug screening assay (16). We have extended these observations and applied an HIV-1 RNA·RNA hybridization assay to measure the relative sensitivity of clinical HIV strains to antiviral agents. The interpretation of results from an *in vitro* system designed to detect HIV resistance to antiviral agents must be undertaken with caution because circumstances of HIV infection can be readily manipulated in the laboratory and may bear no relation to clinical infection. Intracellular HIV-1-specific RNA levels should accurately reflect the transcriptional activity of HIV-infected cells in the presence of an antiviral

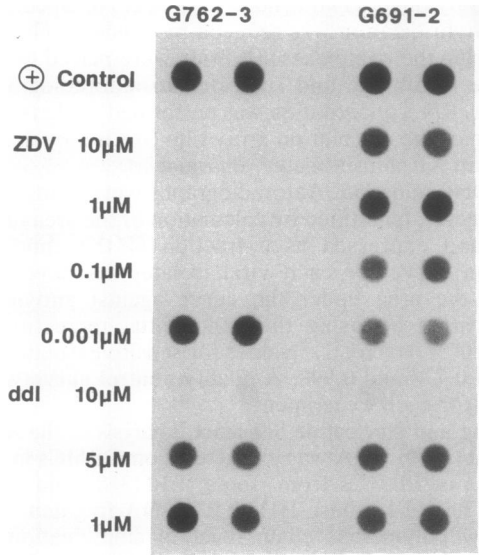

FIG. 3. Twenty-four-hour autoradiograph measuring HIV-1 RNA from 2×10^5 PBMCs following acute infection with HIV strain G762-3 (pre-ZDV isolate) or G691-2 (post-ZDV isolate) and a 7-day incubation in the presence of various concentrations of ZDV or ddI.

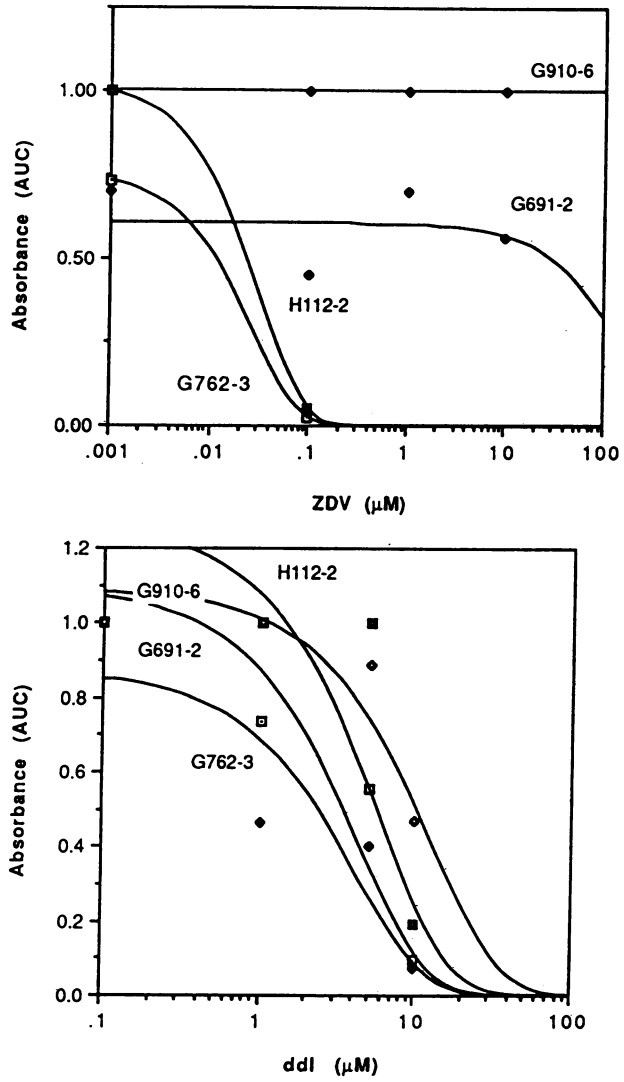

FIG. 4. Sensitivity of HIV-1 isolates to ZDV (*Upper*) and ddI (*Lower*) by quantitation of HIV-1 RNA in the presence of increasing concentrations of drug as described in Fig. 2. AUC, area under the curve.

agent. We chose human PBMCs in our *in vitro* system rather than a continuous cell line because the PBMC coculture consensus method can isolate HIV-1 from blood in >90% of patients, regardless of the stage of illness (17, 18). Other methods using continuous cell lines have been shown to isolate HIV-1 in only 30% of patients (12). Moreover, we believe drug sensitivity assays using PBMCs are more likely to maintain the heterogeneous population of HIV strains *in vitro* and that rapidly growing continuous cell lines may select out HIV strains with tropism for the cell line and may not necessarily be reflective of the entire population of HIV strains present in the patient.

The ZDV IC_{50} for $HTLV\text{-}III_B$ measured by this assay was 0.01 μM and is consistent with published values using other methods (12, 19). To confirm that our method distinguishes ZDV-sensitive from ZDV-resistant isolates and because there is no consensus procedure for the determination of HIV-1 strains resistant to antiviral agents, we tested reference pairs of HIV-1 strains that have been published to be ZDV-sensitive/resistant by an independent method. The reference ZDV-sensitive HIV-1 strains showed a ZDV IC_{50} similar to that of wild-type HIV-1. The reference ZDV-resistant strains showed a ZDV IC_{50} of >10.0 μM. Drug-induced cytotoxicity prevented precise end-point ZDV-sensitivity determination testing on the reference resistant isolates.

In all three clinical isolates from our patients who had had ZDV therapy for greater than 10 months, decreased susceptibility to ZDV was shown. Clinical deterioration was seen in one of two patients with high-level ZDV resistance. In contrast, in two patients without previous ZDV therapy, the ZDV IC_{50} was within the range of the wild-type and control values. In another case, despite withdrawal of ZDV and after 12 weeks of therapy with ddI, the resistant strain was still present. These results confirm that chronic ZDV therapy frequently results in the emergence of highly ZDV-resistant HIV strains. Furthermore, discontinuance of the drug may not result in the disappearance of the resistant HIV strain.

The mechanism(s) of clinical ZDV resistance in HIV-1 remain unclear; however, one hypothesis suggests that mutations in the HIV-1 RT may be responsible for this phenomenon. We confirmed that mutations in the HIV-1 RT gene were associated with resistance in our RNA·RNA hybridization assay by sequencing the complete HIV-1 RT from one ZDV-resistant strain (EP113090). We found 18 nucleotide mutations resulting in eight amino acid changes. Published sequences for wild-type HIV-1 show conservation in four out of the eight amino acid residues (Leu-125 → Trp, Ile-142 → Val, Thr-215 → Tyr, and Pro-294 → Thr) (20). These four amino acids are located close to, but not strictly within, the highly conserved regions of the RT gene (Fig. 5). Larder and Kemp (13) found RT amino acid changes at positions 67, 70, and 215 common to all five resistant strains they analyzed. Our resistant strain did share the Thr-215 → Tyr change but did not contain the other changes. Thr-215 → Tyr requires the rare occurrence of two consecutive nucleotide mutations, and it has been speculated that Thr-215 → Tyr may be the

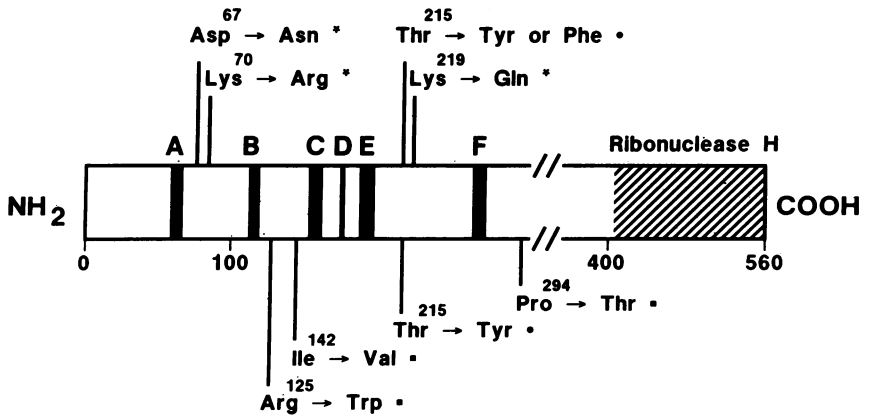

FIG. 5. HIV-1 RT enzyme showing the relationship of highly conserved regions (A–F) among retroviruses and amino acid changes found in EP113089 (■), in resistant strains found by Larder and Kemp (*) (13), and in mutations shared by both analyses (●).

important precursor in the development of ZDV resistance. The other three RT residue changes we found at positions 125, 142, and 294 have not been reported to be associated with ZDV resistance. Since the observations of both groups are based on a small number of clinical ZDV-resistant isolates that have been fully sequenced, more resistant isolates must be identified and analyzed before genotypic mutations associated with ZDV resistance can be conclusively confirmed.

Cross-resistance between ZDV and another closely related pyrimidine nucleoside analogue, 3′-azido-2′,3′-dideoxyuridine, has been shown (12). Two ZDV-resistant isolates have been shown to have elevated RT activity in the presence of dideoxycytidine and ddI compared to ZDV-sensitive strains (21). The reference ZDV-resistant strains we tested appeared to have 2-fold to 5-fold increases in the ddI IC_{50} compared to HTLV-III_B. One of our clinical ZDV-resistant isolates had a 5-fold increase in the ddI IC_{50} compared to HTLV-III_B. Interestingly, this patient, exhibiting decreased sensitivity to ZDV and ddI, was clinically deteriorating, possibly owing to the emergence of a more pathogenic HIV strain. We cannot conclusively state that the use of ZDV selected for cross-resistance to ddI because an HIV isolate predating ZDV therapy was not available for comparison, but the post-ZDV isolate is markedly less sensitive to ddI than the HTLV-III_B strain. Current high-dose ddI regimens at 12.0 mg per kg per day achieve peak plasma ddI levels of 6.3–9.6 μM, which is below the level to inhibit 50% of the RNA production by our less-sensitive ddI strain (22). Potential cross-resistance between RT inhibitors needs to be further investigated since it has an important bearing on the design of future clinical protocols.

In summary, we have designed a system employing an RNA·RNA hybridization procedure that measures the relative sensitivity of clinical HIV isolates to nucleoside analogues. This method should be applicable to rapid testing of all clinical isolates of HIV that are able to grow in PBMCs. This method should also be useful for extensive testing of isolates from patients enrolled in clinical trials evaluating retroviral therapy and permit the correlation of *in vitro* HIV resistance with clinical outcome.

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