Antimicrobial Agents and Chemotherapy, Oct. 1996, p. 2404–2409 Vol. 40, No. 10
0066-4804/96/$04.00+0
Copyright © 1996, American Society for Microbiology

Human Immunodeficiency Virus Type 1 Drug Susceptibility Determination by Using Recombinant Viruses Generated from Patient Sera Tested in a Cell-Killing Assay

CHARLES A. B. BOUCHER,[1,2]* WILCO KEULEN,[1,2] TOON van BOMMEL,[1,2] MONIQUE NIJHUIS,[1,2] DORIEN de JONG,[1,2] MENNO D. de JONG,[2] PAULINE SCHIPPER,[1,2] and NICOLE K. T. BACK[2]

Department of Virology, Eijkman-Winkler Institute, University Hospital Utrecht, 3584 CX Utrecht,[1] and Antiviral Therapy Laboratory, Department of Virology, University of Amsterdam/Academic Medical Centre, Amsterdam,[2] The Netherlands

Received 20 February 1996/Returned for modification 4 April 1996/Accepted 27 June 1996

A simple approach for the determination of drug susceptibilities by using human immunodeficiency virus type 1 (HIV-1) RNA from the sera of patients is described. HIV-1 RNA was extracted from patient sera, and the 5′ part of the reverse transcriptase (RT) gene was transcribed into DNA and amplified in a nested PCR. The amplified fragment covers the 3′ part of the protease gene and amino acids 1 to 304 of the RT gene. This fragment can be introduced through homologous recombination, as described previously, into a novel HIV-1 reference strain (pHXB2Δ2-261RT) from which amino acids 2 to 261 of RT have been deleted. The resulting recombinant virus expresses all properties of the HXB2 reference strain except for those encoded by the introduced part of the patient RT gene. Recombinant viruses were subsequently tested for drug susceptibility in a microtiter format killing assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay] as well as in the standard HeLa CD4$^+$ plaque reduction assay. Similar susceptibility profiles were obtained by each assay with recombinant viruses derived from patients receiving alternating nevirapine and zidovudine treatment or lamivudine-zidovudine combination therapy. In conclusion, this approach enables high-throughput determination of the drug susceptibilities of serum RNA-derived RT genes, independent of the patient's viral background, and generates the possibility of relating changes in susceptibility to changes in viral genotypes.

During treatment of human immunodeficiency virus type 1 (HIV-1)-infected patients with inhibitors of the viral enzymes reverse transcriptase (RT) and protease, resistant viruses are selected. Various biological methods have been developed to assess the drug susceptibility of HIV-1 (2, 5, 8). In most cases these assays require cocultivation of patient peripheral blood mononuclear cells (PBMCs) with donor PBMCs to obtain a viral stock. Subsequently, the drug susceptibility of the viral stock can be determined by the addition of a standardized inoculum to indicator cells in the presence of increasing drug concentrations. In PBMC-based assays, replication of the virus in the presence of increasing drug concentrations is measured by either the production of HIV-1 p24 antigen or RT activity in the culture supernatants. In the HeLa CD4$^+$ plaque reduction assay, the reduction in the number of plaques is measured. Recently, Kellam and Larder (6) described an innovative method of generating viruses for drug susceptibility testing. Their recombinant virus assay consists of amplification of a region of the RT gene from patient cells by PCR. Subsequently, this RT fragment is cotransfected with a molecular clone (HIV-1 HXB2), which lacks a part of the RT gene (amino acids 41 to 523). The resulting recombinant viruses derive all their biological properties from the molecular clone HIV-1 HXB2 except for RT, which is partially encoded by the RT gene from the patient isolates. Since HIV-1 HXB2 induces syncytia in CD4-transfected HeLa cells, the drug susceptibilities of the recombinant viruses can easily be tested in the CD4$^+$ plaque reduction assay. However, large numbers of samples cannot easily be analyzed by the plaque reduction assay, since the number of plaques must be scored. Recombinant viruses generated with the HXB2 molecular clone have good cell-killing capacities induced by the envelope properties of the molecular clone. Therefore, we investigated the possibility of testing the drug susceptibilities of recombinant viruses in a cell-killing assay as described by Pauwels et al. (10). This cell-killing assay measures the capacity of a virus to induce lysis of target cells. Living cells convert 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) into a blue product (formazan). The amount of formazan reflects the number of cells protected by the drug against killing by the virus. The production of formazan can be quantified spectrophotometrically. This, in combination with the possibility of performing the assay in microtiter plates, facilitates testing of large numbers of viral isolates.

In order to be able to measure drug susceptibility from RT genes present in serum, we developed a sensitive approach for the amplification of HIV RNA, combining the reverse transcription and first amplification step in one tube; this is followed by a second (nested) PCR (9). We constructed a new HXB2 deletion plasmid which lacks a smaller region of RT (amino acids 2 to 261) than the original plasmid; this region contains all mutations known to confer drug resistance described in patients.

This paper describes the results of the RNA recombinant virus MTT approach. Serum samples from patients in clinical trials were used to generate the RT fragment. The recombinant viruses were made with the new deletion plasmid. By

* Corresponding author. Mailing address: Department of Virology, Eijkman-Winkler Institute, University Hospital Utrecht, Heidelberglaan 100, 3584 CX Utrecht, The Netherlands. Phone: 31-30-2506526. Fax: 31-30-2541770. Electronic mail address: c.boucher@lab.azu.nl.

using these fragments, recombinant viruses were tested both in the HeLa CD4$^+$ plaque reduction assay and the MTT assay. Finally, susceptibility profiles were compared with the RT sequences obtained by population sequence analysis of the amplified RT genes used to create the recombinant viruses.

MATERIALS AND METHODS

Cell lines. SupT1 cells were used to propagate the molecular clones and were used in transfection experiments to generate the recombinant viruses. MT2 cells were used to calculate the 50% tissue culture infective doses for the MTT sensitivity assay. HT4LacZ-1 is a HeLa cell line expressing CD4 molecules on the surface and β-galactosidase (11) and can therefore be infected with HIV-1 and used for drug susceptibility assays. HT4LacZ-1 cells were used to titrate the virus for the HeLa CD4$^+$ plaque reduction assay and to determine drug susceptibilities (8). All cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (Gibco BRL, Life Technologies Inc., Breda, The Netherlands) and antibiotics. Cell lines were used in the logarithmic phase of growth. After 20 passages, the cells were discarded and new cells were obtained from our repository.

Drug susceptibility assays. Susceptibility to zidovudine (ZDV), lamivudine (3TC), and nevirapine (NVP) were determined in the HeLa CD4$^+$ plaque reduction assay as described previously by Larder et al. (8) by using HT4LacZ cells (11). The MTT assay was performed as described by Pauwels et al. (10). Briefly, the cells were infected with the virus in the presence of increasing drug concentrations. Living cells convert MTT into a blue product, formazan. The amount of formazan reflects the number of cells protected by the drug. A few modifications were introduced. MT2 cells were used instead of MT4 cells, and a cell-free virus titration was performed simultaneously with each susceptibility measurement in order to evaluate the variation in the amount of cell killing caused by a variation in the amount of input virus.

Reference viral strains. The infectious molecular clone HIV-1 HXB2 (containing no ZDV resistance mutations) and HIV-1 RTMC (a gift from B. Larder) containing ZDV resistance-conferring amino acid changes at four codons (aspartic acid to asparagine at codon 67 [D67N], K70R, T215F, and K219Q) introduced in the background of HXB2 by mutagenesis were used to evaluate the MTT assay.

Clinical samples. Serum samples and uncultured PBMCs were obtained from patients participating in two clinical trials. In one trial (Boehringer trial 881) patients were treated with a regimen of NVP (1 week) alternated with ZDV (3 weeks) (4). In the other trial (NUCB trial 2001), patients were treated with 3TC monotherapy and subsequently with a combination of 3TC and ZDV (14).

Nucleic acid isolation. A volume of 100 μl of serum or a pellet of 10^6 uncultured patient PBMCs was used for nucleic acid isolation. RNA and DNA were extracted as described by Boom et al. (1) by a method based on a guanidinium-thiocyanate lysis buffer and glass-milk.

Generation of the HIV-1 RT cDNA fragments by one-tube RT-PCR. Copy DNA fragments were generated by a one-tube RT-PCR approach as described by Nijhuis et al. (9). In this system reverse transcription and amplification were performed in heat-stable microtiter plates (HI TEMP; Techne Inc., Princeton, N.J.) with a thermal cycler (PHC-3; Techne Inc.). Each RT-PCR mixture contained 10 pmol of oligonucleotides RT18 (5′-GGA AAC CAA AAA TGA TAG GGG GAA TTG GAG G-3′ [nucleotides 2376 to 2406]) and RT21 (5′-CTG TAT TTC TGC TAT TAA GTC TTT TGA TGG G-3′ [nucleotides 3538 to 3508]) (Pharmacia Biotech, Roosendaal, The Netherlands), 200 μM (each) the four deoxynucleoside triphosphates (Pharmacia, Piscataway, N.J.), 2 mM $MgCl_2$, 67 mM Tris-HCl (pH 8.8), 17 mM $(NH_4)_2SO_4$, 1 mM β-mercaptoethanol, 6 μM EDTA, 400 ng of poly(A) RNA (Pharmacia), 0.2 mg of bovine serum albumin (Boehringer, Mannheim, Germany) per ml, 2.5 U avian myeloblastosis virus RT (Boehringer), and 1.7 U of AmpliTaq polymerase (Perkin-Elmer, Norwalk, Conn.). All reactions were performed in a 50-μl volume and were covered with paraffin oil. The reaction mixtures were incubated for 30 min at 42°C to enable cDNA synthesis. Afterward the RT enzyme was inactivated (5 min at 95°C) and cDNA was amplified for 35 cycles by the following protocol: 1 min at 95°C, 1 min at 55°C, and 2 min at 72°C. Subsequent to this procedure the amount of amplified product was further increased in a second (nested) amplification reaction. A total of 5 μl of the RT-PCR mixture was transferred to a fresh (50-μl) amplification mixture containing 14 pmol of each of the (nested) oligonucleotides RT19 (5′-GGA CAT AAA GCT ATA GGT ACA G-3′ [nucleotides 2453 to 2474]) and RT20 (5′-CTG CCA GTT CTA GCT CTG CTT C-3′ [nucleotides 3461 to 3440]) (Pharmacia Biotech) and the same reaction buffer described for the RT-PCR except for the presence of poly(A) RNA and avian myeloblastosis virus RT. In order to reduce the chance that a contaminant of the second PCR would be amplified to a detectable concentration, the nested amplification reaction consisted of 25 instead of 35 cycles. After optimization the sensitivity of the entire (nested) procedure was as low as 50 molecules of HIV-1 RNA.

Construction of an RT-deleted proviral molecular clone (pHXB2Δ2-261RT). The deletion clone was constructed from an HXB2 molecular clone. In this clone a deletion in the HXB2-RT gene of 260 amino acids (amino acids 2 to 261 of RT) was introduced. The *Sma*I site present in the vector pSP64 containing the molecular clone HXB2ΔBstEII (gift of Brendan Larder [6]) was removed (HXB2ΔBstEII/SmaI$^-$). For the construction of the RT deletion clone, a PCR was performed with PFU polymerase (Stratagene, Westburg bv, Leusden, The Netherlands) on HXB2 with primers SK145 (nucleotides 1358 to 1387; 5′-AGT GGG GGG ACA TCA AGC AGC CAT GCA AAT-3′) and 3′Sma-RT (nucleotides 2565 to 2535; 5′-GGG GGT CAC CCC CGG GAA AAT TTA AAG TGC-3′). This PCR fragment was digested with *Apa*I and *Sma*I and was cloned into pGem 7(+) (Promega, Leiden, The Netherlands). The resulting clone, 5′Sma-RT/7, contained the HXB2 sequence from positions 2009 to 2551. A second PCR fragment was generated with the primers 5′Sma-RT (nucleotides 3326 to 3346; 5′-TTA CCC GGG AAA TTG AAT TGG-3′) and 3′Pol-MX (nucleotides 5016 to 4982; 5′-CTT GGA TCC ACT ACG CGT ATG TCA CTA TTA TCT TG-3′). This fragment was digested with *Sma*I and *Mlu*I and cloned into the 5′Sma-RT/7 clone. The resulting construct, small ΔRT/7, contained the HXB2 sequence from positions 2009 to 2551 and from positions 3332 to 5098. Both the small ΔRT/7 clone and the HXB2ΔRTBstEII/SmaI$^-$ clone were transformed into *Escherichia coli* dam$^-$ cells to change the methylation status of the DNA. Through *Bcl*I (position 2428) and *Bal*I (position 4552) digestion the unique *Sma*I site was introduced into the HXB2ΔRTBstEII/SmaI$^-$ clone, resulting in pHXB2Δ2-261RT.

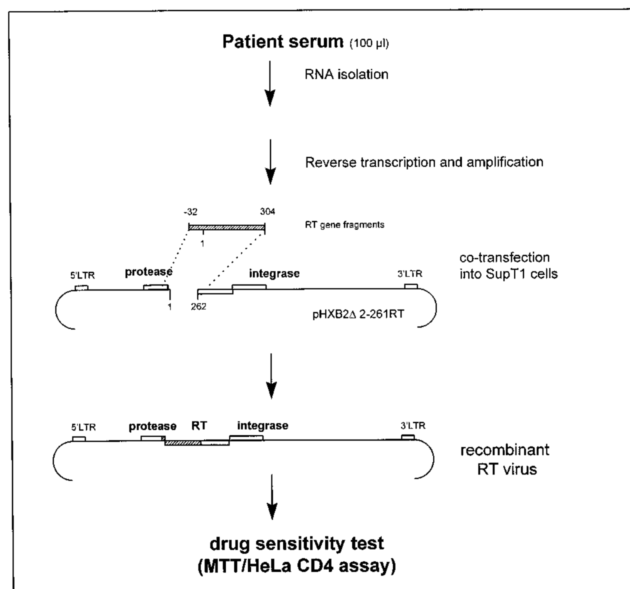

FIG. 1. Approach used to test drug susceptibilities using recombinant viruses containing RT genes present in serum samples from treated patients. After extraction of the viral RNA, a part of the protease gene and the amino-terminal part of the RT gene was reverse transcribed and PCR amplified in an one-tube reaction. The amount of product was further increased by a second PCR. The resulting PCR fragment containing the first 261 amino acids of the RT gene, and the flanking sequences was cotransfected with a molecular clone lacking amino-terminal amino acids 2 to 261. Because of the presence of the overlapping sequences, homologous recombination occurs and the recombinant virus contains amino acids 2 to 261 from the patient sample. The recombinant virus can be tested in either the HeLa CD4$^+$ plaque reduction assay or the MTT assay. LTR, long terminal repeat.

Electroporation of the HIV-1 RT fragments. The amplified DNA products were purified by a phenol-chloroform extraction and ethanol precipitation, and the amount of product was determined spectrophotometrically. When the source of patient material was DNA from cells, 1 μg of the PCR fragment was cotransfected by electroporation with 1 μg of the deletion clone pHIVΔRTBstEII (linearized by a *Bst*EII digestion). When patient serum was used as the source, 1 μg (linearized by *Sma*I digestion) of pHXB2Δ2-261RT was cotransfected with 1 μg of the PCR-amplified cDNA product. Electroporation procedures were as follows: DNA fragments were cotransfected in 5×10^6 SupT1 cells with a Bio-Rad gene pulser (250 V; 960 μF) (3). After electroporation 0.5×10^6 fresh SupT1 cells were added, and the volume was expanded to 5 ml with culture medium. The cultures were monitored daily for syncytium formation, and fresh medium was added to maintain an optimal cell concentration of approximately 0.2×10^6 cells per ml. Viral supernatants were harvested by centrifugation (10 (1,000 × *g* for 10 min) when the culture contained 100% full-blown syncytia, which indicates that nearly all cells in the culture are involved in giant cell formation. Viral stocks were stored at −70°C for subsequent titration and sensitivity testing.

TABLE 1. Comparison of MTT assay with plaque reduction assay with defined molecular clones (HXB2 and RTMC containing four ZDV resistance mutations)

Clone	Amino acid at position: 67	70	215	219	Assay	Geometric mean IC_{50} of ZDV (μM [range])	Fold increase in IC_{50}
HXB2	D	K	T	K	MTT	0.11 (0.07–0.16)	
					Plaque	0.07 (0.04–0.12)	
RTMC	**N**[a]	**R**	**F**	**Q**	MTT	6.4 (2.6–15.7)	58
					Plaque	2.0 (1.12–3.56)	29

[a] Boldface type indicates amino acid changes.

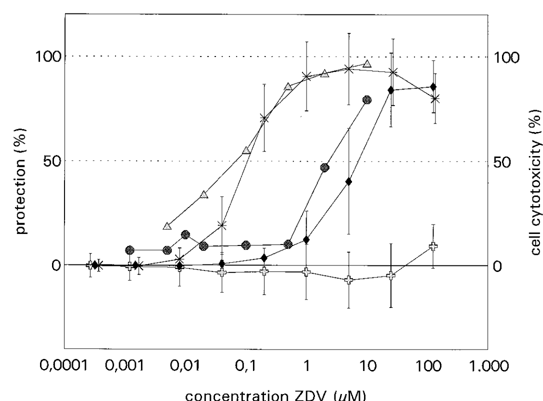

FIG. 2. Susceptibility curves for each ZDV concentration for molecular clones HXB2 and RTMC in two susceptibility assays. For the MTT assay the standard deviations are shown. In addition, the toxic effect of ZDV on MT2 cells, as measured in the MTT assay, is shown. Symbols: △, HXB2 (plaque assay); *, HXB2 (MTT assay); ●, RTMC (plaque assay); ◆, RTMC (MTT assay); ⊕, 2DV toxicity.

A schematic representation of the construction of recombinant viruses from serum is shown in Fig. 1.

Direct sequencing of patient cDNA fragments from serum HIV-1 RNA. Amplified cDNA products were obtained by the one-tube RT-PCR approach. For the samples obtained from patients participating in the Boehringer study, two separate nested PCR amplifications were performed with primer pair RT19 with biotinylated K-RT (nucleotides 3257 to 3240; 5′-CAG GAT GGA GTT CAT AAC-3′) and biotinylated RT19 with K-RT. This resulted in amplification products of which one of the strands was biotinylated. Subsequently, single-stranded DNA was purified by using polystyrene streptavidin-coated magnetic beads (Dynabeads M280; Dynal AS, Oslo, Norway). Single-stranded DNA was sequenced by the dideoxy chain termination method with Sequenase 2.0 (United States Biochemicals, Cleveland, Ohio), with modifications as described previously (12). The use of primers K-RT, BR-RT (nucleotides 3018 to 3002; 5′-GGT GAT CCT TTC CAT CC-3′), C-RT (nucleotides 2645 to 2664; 5′-GCA TTA GTA GAA ATT TGT AC-3′), and RT19 enabled sequence analysis of HIV-1 RT amino acids 1 to approximately 225.

For the analysis of samples obtained from patients participating in the NUCB 2001 trial, we used two different nested PCR products. The primer pairs biotinylated RT19 with RT20 and biotinylated RT20 with RT19 were used in the nested PCR. For sequencing, the primers K-RT, BR-RT, RT-11 (nucleotides 3110 to 3128; 5′-TAT GTA GGA TCT GAC TTA G-3′), and 70-PMA (nucleotides 2758 to 2774; 5′-CTA CTA ATT TTC TCC AT-3′) were used. Use of these primers enabled the analysis of the sequence of amino acids 1 to approximately 262.

RESULTS

Comparison of the cell killing (MTT) assay with the plaque reduction (HeLa $CD4^+$) assay with HIV-1 ZDV-susceptible and -resistant viruses derived from molecular clones. Since the MTT assay measures the amount of cells killed by the virus, it is very sensitive to variation in the amount of viral input. We therefore determined the effect of variations in the multiplicity of infection (MOI) and observed that the MOI should range between 1×10^{-3} and 5×10^{-3}: i.e., 400 to 2,000 50% tissue culture infective doses per 0.4×10^6 MT2 cells. Generally, the best results were obtained with an MOI of 3×10^{-3} (data not shown).

Storage of a viral stock and thawing may introduce significant changes in viral titer. Consequently, we decided to repeat the MOI determination in parallel with each drug susceptibility assay.

The ratio between the mean optical densities of uninfected and infected cells was used to determine the viral input. With a viral input of approximately 10^{-3} MOI, this ratio needs to be between 5 and 20.

In order to evaluate the MTT assay, two well-characterized molecular HIV-1 clones, HXB2 with no ZDV mutations and HXB2-RTMC containing four ZDV resistance mutations, were tested (Table 1). For both the MTT and the plaque reduction assays we determined the geometric means of the 50% inhibitory concentrations (IC_{50}s) for ZDV and the fold increase in the IC_{50} (Table 1). The range of IC_{50}s in the MTT assay observed with HXB2 was between 0.07 and 0.16 μM (10 measurements); the range of IC_{50}s observed with RTMC was 2.6 to 15.7 μM (four measurements). The ranges of IC_{50}s in the HeLa $CD4^+$ plaque reduction assay were 0.04 to 0.12 μM with HXB2 and 1.12 to 3.56 μM with RTMC (Table 1). The variations in the MTT assay and in the plaque assay for the susceptible clone HXB2 were 0.4 and 0.5 log, respectively. The fold increase in the IC_{50} was significant (1.5 to 1.8 log) and was of the same order of magnitude in both assays. Figure 2 shows the susceptibility curves for each ZDV concentration for both viruses in each assay. The toxicity of ZDV for the MT2 cells measured in the MTT assay is also shown.

Comparison of the MTT assay with the plaque reduction assay with recombinant viruses derived from proviral DNA isolated from patient cells. From six patients receiving an alternating treatment of NVP and ZDV (trial 881), paired PBMC samples were obtained and analyzed twice in both susceptibility assays at the start and after 16 weeks of treatment (Table 2). The NVP IC_{50}s for viruses from untreated individuals in the plaque reduction assay ranged from 0.02 to 0.09 μM (variation, 0.6 log), and in the MTT assay the IC_{50}s ranged from 0.03 to 0.27 μM (variation, 1 log). The IC_{50}s of ZDV in the plaque reduction assay ranged from 0.05 to 0.09 μM (variation 0.3 log), and in the MTT assay the IC_{50}s ranged from 0.08 to 0.36 μM (variation, 0.7 log). The intra-assay variation in IC_{50}s for the two susceptibility assays with the same stock of recombinant viruses was 0.6 and 1 log, respectively, for NVP and 0.3 and 0.7 log, respectively, for ZDV. The increases in the IC_{50}s observed for viruses from patients between week 0 and week 16 in both assays were comparable. For viruses from four of six patients, a significant increase in the IC_{50} of NVP was found after 16 weeks of alternating therapy (1.4 to 3 log) in both assays. Only for virus from one patient (patient N240) was a significant increase in the IC_{50} of ZDV shown after 16 weeks of alternating treatment.

Comparison of the MTT assay with the plaque reduction assay with recombinant viruses derived from viral RNA isolated from patient sera. From three of the six patients receiving the alternating regimen of NVP and ZDV (Table 2), paired serum samples obtained at the start and after 16 weeks of therapy were analyzed in both susceptibility assays (Table 3). The range of IC_{50}s of NVP observed for viruses from untreated individuals in the plaque reduction assay was from 0.02 to 0.08 μM, and that in the MTT assay was from 0.04 to 0.13 μM. The IC_{50}s of ZDV in the plaque reduction assay ranged from 0.05

TABLE 2. Comparison of MTT assay with plaque reduction assay for proviral population (geometric mean of duplicate experiments) obtained from DNA in patient PBMCs

Patient no.	Wk	Assay	NVP		ZDV	
			Geometric mean IC_{50} (μM)	Fold increase in IC_{50}	Geometric mean IC_{50} (μM)	Fold increase in IC_{50}
N233	0	MTT	0.18		0.14	
		Plaque	0.06		0.10	
	16	MTT	0.57	3	0.07	<1
		Plaque	0.58	10	0.10	1
N240	0	MTT	0.02		0.07	
		Plaque	0.04		0.05	
	16	MTT	>10	>500	1.28	18
		Plaque	>10	>250	0.2	4
N241	0	MTT	0.20		0.27	
		Plaque	0.08		0.08	
	16	MTT	5.37	27	0.22	<1
		Plaque	2.02	25	0.04	<1
N242	0	MTT	0.36		0.26	
		Plaque	0.08		0.08	
	16	MTT	>10	>28	0.20	<1
		Plaque	5.74	72	0.11	1
N243	0	MTT	0.04		0.27	
		Plaque	0.01		0.06	
	16	MTT	>10	>250	0.11	<1
		Plaque	>10	>100	0.07	1
N244	0	MTT	0.04		0.17	
		Plaque	0.04		0.04	
	16	MTT	0.04	1	0.12	<1
		Plaque	0.03	<1	0.04	1

to 0.07 μM, and in the MTT assay they ranged from 0.05 to 0.10 μM. The geometric mean IC_{50}s in both assays are presented in Table 3. The intra-assay variations in IC_{50} by the two susceptibility assays (the plaque reduction and the MTT assays) with the same stock of recombinant viruses were 0.6 and 0.5 log, respectively, for NVP and 0.2 and 0.4 log, respectively, for ZDV. For all three recombinant viruses obtained after 16 weeks of therapy, a significant increase in the IC_{50} of NVP was found in both assays (1.3 to 2.4 log); no significant changes in the IC_{50}s of ZDV were found. Sequence analysis of the RNA samples used to generate the recombinant viruses revealed the presence of several of the previously described nonnucleoside RT inhibitor resistance mutations. Codon changes conferring ZDV resistance were observed for only one patient, for whom a mixed population for codon 70 was detected.

Recombinant viruses were obtained from the serum of two patients initially treated for 1 year with 3TC monotherapy and subsequently with a combination of 3TC with ZDV. A difference in the absolute IC_{50}s was observed between the MTT assay and the plaque reduction assay (Table 4) for recombinant viruses which had been obtained before the initiation of therapy. During 3TC therapy, viruses with a highly 3TC-resistant phenotype developed in both patients. We were unable to calculate the IC_{50}s for the viruses because 50% inhibition was not achieved with the highest drug concentrations that can be used in our assay without cell toxicity. The addition of ZDV therapy to the 3TC therapy for 1 year resulted in the appearance of viruses which also showed low levels of ZDV resistance.

DISCUSSION

By combining previously described techniques, we developed an innovative approach to evaluate the appearance of drug-resistant viruses in serum from HIV-1-infected patients treated with RT inhibitors. One of our objectives was to develop an approach to measure the drug susceptibility of the viral population instead of the proviral population. The development of the drug-resistant genotypes in serum precedes the appearance of drug-resistant proviruses in the patients' PBMCs (7, 13). Moreover, it appears that the appearance of drug-resistant viral genotypes in serum is closely associated in time with the resurgence in viral HIV-1 RNA load during therapy. In a patient group on 3TC monotherapy, we were able to show that the resurgence in HIV-1 RNA levels after the initial decline was solely due to the replication of viruses with an amino acid change at codon 184 (13). In contrast, when we analyzed the proviral population in these individuals over time, we observed a persistence of the drug-susceptible virus with the wild-type amino acid at codon 184. These data indicate that to relate viral RNA patterns in serum with the development of drug-resistant viruses, it is mandatory that the drug susceptibility of the viral population in serum be measured.

The recovery of viruses by cocultivation from serum is extremely difficult; therefore, we chose to generate RT fragments from the patient's serum and introduce them into the background of an HIV-1 reference strain using a modification of the recombinant virus approach described by Kellam and Larder (6). Their deletion clone, originally used for the recombinant assay, still contains amino acids 1 to 41 of the RT at its 5′ end. Since changes at codon 41 are involved in conferring resistance to ZDV, we decided to design a deletion clone starting at codon 1 of the RT gene. Moreover, recovery of HIV-1 RNA from serum becomes more efficient when shorter fragments are transcribed and amplified. Therefore, we decided to construct a deletion clone which requires amplification of a shorter fragment of approximately 1 kb. The recombinant virus generated with this novel deletion clone (pHXB2Δ2-261 RT) contains amino acids 2 to 261 of the RT from the patient's viral population.

This region contains all known resistance-conferring amino acid changes described in viruses from patients treated with nucleoside analogs and from patients treated with nonnucleoside RT inhibitors. However, the possibility remains that new codon changes causing resistance outside this region will be found. Especially with the use of new drugs or new combinations of available drugs, new mutations which are located 3′ from amino acid 261 could start to play a role. This may limit the use of the assay in its present form, and therefore, we are modifying the approach in such a way that the entire RT region, possibly in combination with the neighboring protease region, can be derived from patient serum.

The MTT assay described by Pauwels et al. (10) has the advantage that the amount of cell killing by the virus can be determined semiautomatically with a spectrophotometer. This generates the possibility that large numbers of viruses can be

TABLE 3. Comparison of MTT assay with plaque reduction assay for viral population (geometric mean of duplicate experiments) obtained from RNA in serum and genotypic changes observed in serum

Patient no.	Wk	Amino acid at position:							Assay	NVP		ZDV	
		70	98	100	101	103	188	190		Geometric mean IC_{50} (μM)	Fold increase in IC_{50}	Geometric mean IC_{50} (μM)	Fold increase in IC_{50}
N233	0	K	S	L	K	K	Y	G	MTT	0.07		0.08	
									Plaque	0.03		0.07	
	16					**N**[a]			MTT	>10	>143	0.24	3
									Plaque	7.59	253	0.04	1
N241	0	K	A	L	K	K	Y	G	MTT	0.13		0.07	
									Plaque	0.09		0.06	
	16	**k/R**				**k/N**	**y/H**	**g/A**	MTT	8.13	63	0.05	<1
									Plaque	>10	>111	0.06	1
N243	0	K	A	L	K	K	Y	G	MTT	0.03		0.05	
									Plaque	0.02		0.06	
	16		**a/P**	**l/F**	**k/R**	**k/N**		**A**	MTT	2.40	80	0.14	3
									Plaque	0.39	20	0.06	1

[a] Boldface type indicates amino acid changes.

analyzed. However, the assay is limited by the facts that variations in the viral titer and in the viral capacity to kill cells can bias the susceptibility measurements. Especially when testing clinical isolates, it is difficult to control for these variabilities. These problems can be overcome by making use of the recombinant virus approach. First, the cell-killing capacity of the virus is merely determined by the viral envelope. The recombinant viruses contain a constant envelope derived from the deletion clone. Second, the replication capacities of recombinant viruses will also be largely determined by the deletion clone and therefore make it easy to obtain a high viral titer with good T-cell-killing capacity.

TABLE 4. Comparison of MTT assay with plaque reduction assay for viral population (geometric means of duplicate experiments) obtained from RNA in serum

Patient no. and therapy	Assay	3TC		ZDV	
		Geometric mean IC_{50} (μM)	Fold increase in IC_{50}	Geometric mean IC_{50} (μM)	Fold increase in IC_{50}
C0011					
None	MTT	7.35		0.06	
	Plaque	0.22		0.04	
3TC for 71 wk	MTT	>1,000	>136	0.08	1
	Plaque	>100	>455	0.02	<1
3TC-ZDV for 46 wk	MTT	>1,000	>136	1.2	20
	Plaque	>100	>455	0.25	6.3
C0021					
None	MTT	12		0.11	
	Plaque	0.46		0.08	
3TC for 62 wk	MTT	>1,000	>83	0.14	1
	Plaque	>100	>217	0.06	<1
3TC-ZDV for 48 wk	MTT	>1,000	>83	0.62	6
	Plaque	>100	>217	0.32	4

Comparison of the HeLa plaque reduction assay with the MTT assay with well-defined viral strains as well as recombinant viruses obtained from clinical material showed similar results. Both the absolute IC_{50}s and the fold increase in resistance against two classes of drugs, the nucleoside analog ZDV (which does require phosphorylation by the cells) as well as NVP (which does not require phosphorylation) were comparable in both assays. In the case of testing for susceptibility to 3TC, we have noticed a difference in the absolute IC_{50}s between the two assays. The basis for this difference might be explained by a different phosphorylation metabolism between MT2 and HeLa cells. Despite this difference in absolute values, comparable increases in IC_{50}s during 3TC resistance were observed by using both assays.

In order to obtain good reproducibility it is important that the inoculum in the MTT assay be kept constant (MOI, 1×10^{-3} to 5×10^{-3}), which will result in a ratio of between 5 and 20 in the MTT assay. Therefore, we strongly suggest that titration of the recombinant virus stock be performed before testing susceptibility in the MTT assay. Although we noticed that some variation in viral titer due to storage or thawing, or both, may be observed, as long as the ratio in the MTT assay is between 5 and 20, this will not bias the susceptibility measurement.

In conclusion, the recombinant virus RNA-MTT approach enables measurement of the changes in drug susceptibility directly from RNA in serum. In addition, this assay facilitates the testing of large numbers of viruses. Furthermore, sequence analysis of the amplified fragment used to generate the recombinant virus can be performed in parallel; this enables one to directly relate phenotypic changes to genotypic changes.

ACKNOWLEDGMENTS

We thank B. Larder for providing us with the molecular clones HIV RTMC and the deletion clone pHIVRTΔBstEII, J. de Jong for mo-

lecular clone HXB2, R. Schuurman for critically reading the manuscript, and R. van Leeuwen and S. Danner for providing us with the clinical material.

REFERENCES

1. **Boom, R., C. J. A. Sol, M. M. M. Salimans, C. L. Jansen, P. M. E. Wertheim-van Dillen, and J. van der Noordaa.** 1990. Rapid and simple method for purification of nucleic acids. J. Clin. Microbiol. **28:**495–503.
2. **Brun-Vézinet, F., D. Ingrand, L. Deforges, K. Gochi, F. Ferchal, M.-P. Schmitt, M. Jung, B. Masquelier, J. Aubert, C. Buffet-Janvresse, and H. Fleury.** 1992. HIV-1 sensitivity to zidovudine: a consensus culture technique validated by genotypic analysis of the reverse transcriptase. J. Virol. Methods **37:**177–188.
3. **de Jong, J. J., J. Goudsmit, W. Keulen, B. Klaver, W. Krone, M. Tersmette, and A. de Ronde.** 1992. Human immunodeficiency virus type 1 clones chimeric for the envelope V3 domain differ in syncytium formation and replication capacity. J. Virol. **66:**757–765.
4. **de Jong, M. D., M. Loewenthal, C. A. Boucher, I. van der Ende, D. Hall, P. Schipper, A. Imrie, H. M. Weigel, R. H. Kauffmann, R. Koster, et al.** 1994. Alternating nevirapine and zidovudine treatment of human immunodeficiency virus type 1-infected persons does not prolong nevirapine activity. J. Infect. Dis. **169:**1346–1350.
5. **Japour, A. J., D. L. Mayers, V. A. Johnson, D. R. Kuritzkes, L. A. Beckett, J. M. Arduino, J. Lane, R. J. Black, P. S. Reichelderfer, R. T. D'Aquila, C. S. Crumpacker, The RV-43 Study Group, and the AIDS Clinical Trials Group Virology Committee Resistance Working Group.** 1993. Standardized peripheral blood mononuclear cell culture assay for determination of drug susceptibilities of clinical human immunodeficiency virus type 1 isolates. Antimicrob. Agents Chemother. **37:**1095–1101.
6. **Kellam, P., and B. A. Larder.** 1994. Recombinant virus assay: a rapid, phenotypic assay for assessment of drug susceptibility of human immunodeficiency virus type 1 isolates. Antimicrob. Agents Chemother. **38:**23–30.
7. **Kozal, M. J., R. W. Shafer, M. A. Winters, D. A. Katzenstein, and T. C. Merigan.** 1993. A mutation in human immunodeficiency virus reverse transcriptase and decline in CD4 lymphocyte numbers in long-term zidovudine recipients. J. Infect. Dis. **167:**526–532.
8. **Larder, B. A., B. Chesebro, and D. D. Richman.** 1990. Susceptibilities of zidovudine-susceptible and -resistant human immunodeficiency virus isolates to antiviral agents determined by using a quantitative plaque reduction assay. Antimicrob. Agents Chemother. **34:**436–441.
9. **Nijhuis, M., C. A. B. Boucher, and R. Schuurman.** 1995. Sensitive procedure for the amplification of HIV-1 RNA using a combined reverse-transcription and amplification reaction. BioTechniques **19:**178–180.
10. **Pauwels, R., J. Balzarini, M. Baba, R. Snoeck, D. Schols, P. Herdewijn, J. Desmyter, and E. De Clercq.** 1988. Rapid and automated tetrazolium based colorimetric assay for the detection of anti-HIV compounds. J. Virol. Methods **20:**309–321.
11. **Rocancourt, D., C. Bonnerot, H. Jouin, M. Emerman, and J. F. Nicolas.** 1990. Activation of a β-galactosidase recombinant provirus: application to titration of human immunodeficiency virus (HIV) and HIV-infected cells. J. Virol. **64:**2660–2668.
12. **Schuurman, R., and W. Keulen.** 1990. Modified protocol for DNA sequence analysis using Sequenase 2.0. BioTechniques **11:**185.
13. **Schuurman, R., M. Nijhuis, R. van Leeuwen, P. Schipper, D. de Jong, P. Collis, S. A. Danner, J. Mulder, C. Loveday, C. Christopherson, et al.** 1995. Rapid changes in human immunodeficiency virus type 1 RNA load and appearance of drug-resistant virus populations in persons treated with lamivudine (3TC). J. Infect. Dis. **171:**1411–1419.
14. **van Leeuwen, R., C. Katlama, V. Kitchen, C. A. Boucher, R. Tubiana, M. McBride, D. Ingrand, J. Weber, A. Hill, H. McDade, et al.** 1995. Evaluation of safety and efficacy of 3TC (lamivudine) in patients with asymptomatic or mildly symptomatic human immunodeficiency virus infection: a phase I/II study. J. Infect. Dis. **171:**1166–1171.