ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 1997, p. 2484–2491 Vol. 41, No. 11
0066-4804/97/$04.00+0

Limiting Deoxynucleoside Triphosphate Concentrations Emphasize the Processivity Defect of Lamivudine-Resistant Variants of Human Immunodeficiency Virus Type 1 Reverse Transcriptase

NICOLE K. T. BACK AND BEN BERKHOUT*

Department of Human Retrovirology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands

Received 5 February 1997/Returned for modification 7 April 1997/Accepted 4 August 1997

The nucleoside drug lamivudine (3TC) triggers the selection of resistant forms of the human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) with a substitution of amino acid 184Met. The 3TC-resistant RT enzymes 184Val and 184Ile exhibit a processivity defect in in vitro assays that correlates with reduced replication of the corresponding virus variants in primary cells. However, no replication defect is apparent for these two mutants in the transformed T-cell line SupT1. One obvious difference between the two cell types is the intracellular deoxynucleoside triphosphate (dNTP) level. Primary cells have a much smaller dNTP pool, and this cellular condition may emphasize the processivity defect of the codon 184 RT variants. Alternatively, cell-specific cofactors that influence the process of reverse transcription may exist. Such accessory factors may be packaged into the virion to exert an effect on the RT enzyme. To discriminate between these possibilities we performed additional assays with the wild-type and mutant RT enzymes. The RT proteins were either isolated from virions produced by primary and transformed cell types or expressed as recombinant protein. We also performed infection assays with cells treated with a drug that reduces the intracellular dNTP pool. Furthermore, reverse transcription was studied within virus particles in the endogenous assay, which allows for the manipulation of the dNTP level. The combined results indicate that the enzymatic defect of the 3TC-resistant HIV-1 variants is stressed at low dNTP concentrations.

The clinical use of antiviral compounds targeting the human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) enzyme has been hampered by the ability of the virus to rapidly become resistant by acquisition of one or several amino acid substitutions within the RT protein. Two promising findings indicate that the situation is not completely hopeless. First, there is accumulating evidence that combination therapy leads to a more profound and prolonged suppression of HIV-1 replication, thereby reducing the chances of generating resistant virus variants (4, 12, 19, 39, 41). It is likely that a certain threshold level of virus replication is required to generate appropriate mutations that cause resistance to multiple drugs. In contrast, resistance to a single drug can be provided by an individual amino acid change, and it has been hypothesized that such point mutants are present in the pretreatment virus population of an infected individual (10, 33). Second, there are increasing numbers of reports on functional defects in the variant RT enzymes that cause resistance to nucleoside or nonnucleoside analogs (2, 7, 13, 35). On the basis of the kinetics of HIV-1 replication in infected patients (20, 38, 51), it is likely that even a partially reduced level of virus replication will be of considerable clinical benefit. It has been suggested that a twofold reduction in the number of productively infected cells can lead to a twofold increase in the mean clinical latency (10), although it is obvious that the exact relationship between virus replication and AIDS pathogenesis is unknown.

We previously studied the enzyme and virus replication properties of two HIV-1 RT variants that appear in vitro and in vivo in response to the nucleoside analogue lamivudine (3TC), which is the (−) enantiomer of 2′,3′-dideoxy-3′-thiacytidine (8, 9, 40). In these 3TC-resistant variants the Met184 residue within the catalytic core of the RT enzyme has been changed into either Val or Ile. Both codon 184 variants are frequently observed in treated patients and were demonstrated to provide drug resistance (6, 14, 42, 46, 49). A detailed study indicated that wild-type virus is displaced by the 184Ile variant in most patients after approximately 1 week of 3TC treatment (42). After approximately 2 weeks, another 184Val variant appears, and this virus outgrows the 184Ile variant. Some recent results explain this typical pattern of evolution of drug resistance. First, the HIV-1 RT mutation bias was found to determine the initial appearance of the 184Ile variant (22). Second, defects in the enzymes of both codon 184 variants in comparison with the wild-type RT enzyme were measured. In particular, processivity defects were reported for both 3TC-resistant RT variants, and the activity of the 184Ile enzyme is affected significantly more than the activity of the 184Val enzyme (2). The ranking of the order of RT activity (Met [wild type] > Val > Ile) is consistent with the replication capacity measured in peripheral blood mononuclear cells (PBMCs). These combined results explain both the initial appearance of 184Ile and the eventual outgrowth of 184Val.

In contrast to the replication differences among 3TC-resistant viruses in primary cells, little or no reduction in replication was measured for the 3TC-resistant HIV-1 variants in a transformed T-cell line (2, 24). We suggested that differences in the intracellular concentration of deoxynucleoside triphosphates (dNTPs) cause this cell type-specific defect of the 3TC-resistant viruses. Alternatively, quantitative or qualitative differences may exist in the RT enzyme or the virion particles produced by primary versus transformed cell types. For instance, viral and/or cellular cofactors that affect the activity and/or processivity of the RT enzyme may exist. There is some evi-

* Corresponding author. Mailing address: Department of Human Retrovirology, Academic Medical Center, University of Amsterdam, P.O. Box 22700, 1100 DE Amsterdam, The Netherlands. Phone: (31-20) 5664822. Fax: (31-20) 6916531. E-mail: b.berkhout@amc.uva.nl.

dence that the properties of the HIV-1 RT enzyme are changed in the presence of factors provided by the virion particle (21, 29, 45). The effects on reverse transcription may also be indirect, for instance, through induced differences in the architecture of retroviral particles. This structure, which is designed to optimize reverse transcription, may differ in composition or structure, depending on the host cell type.

We set out to test whether the defect of 3TC-resistant RT variants in primary cells is caused by the low dNTP levels or by a cell-type specific difference in the RT enzyme or virion composition or structure. To discriminate between these possibilities, we compared the in vitro enzyme activities and processivities of the wild-type and 3TC-resistant RT proteins that were isolated from virions produced in either cell type. In addition, we expressed the different RT forms as recombinant proteins in *Escherichia coli*. These experiments indicate that the enzyme defect of 3TC-resistant RT is apparent without specific cellular or viral protein cofactors. To directly test whether the conditional RT defect is triggered by low intracellular dNTP levels, we studied virus replication kinetics in cells treated with a drug that reduces the intracellular dNTP pool. Furthermore, we performed the endogenous reverse transcription assay within wild-type and mutant virion particles in the presence of various amounts of dNTPs. These combined approaches indicate that the replication defect of 3TC-resistant RT variants is confined to primary cell types because of a low dNTP pool.

MATERIALS AND METHODS

Cells, transfection, infection, and virus stocks. Codon 184 of the HIV-1 RT gene (wild-type ATG encodes Met) was replaced by site-directed mutagenesis into either ATA (Ile) or GTG (Val) and was inserted into an HXB2 molecular clone as described previously (2). SupT1 T-cell line and PBMCs were cultured in RPMI medium and transfected and infected as described previously (2). The transfected and infected cells were cultured in the absence or presence of 10-fold dilutions of hydroxyurea (HU; Sigma). Virus stocks were prepared in the transformed T-cell line SupT1 and primary PBMCs were prepared by electroporation (2) with the wild type and mutant molecular clones (5 μg of DNA per 5×10^6 SupT1 cells in a T25 flask and 10 μg per 5×10^6 PBMCs in a T25 flask). When massive syncytia were observed in the SupT1 culture (at approximately day 5) and after 4 weeks of culture in PBMCs, we harvested the supernatant and removed the cells by low-speed centrifugation (10 min, $1,000 \times g$). The supernatant was filtered (pore size, 0.45 μm; Millipore), and a small aliquot (3 ml for SupT1 cells and 5 ml for PBMCs) was stored in liquid nitrogen. The remaining supernatant was used to pellet virion particles by ultracentrifugation (3 h, $100,000 \times g$, 4°C) over a sucrose cushion (20% [wt/vol]). The pellet corresponding to 10 ml of SupT1 culture supernatant was solubilized in 500 μl of RPMI medium (20-fold concentration). The virion stocks from PBMCs were concentrated 60-fold for subsequent Western blot analysis.

Virion and *E. coli* RT enzymes. RT protein was released from virion particles by detergent treatment (Nonidet P-40; final concentration, 0.5% [2]). The wild-type and mutant RT forms were expressed as a glutathione *S*-transferase fusion protein in *E. coli* (36a) and were purified on glutathione beads as described previously (44).

Western blot analysis. Western blotting was performed with concentrated virion samples produced by SupT1 cells and PBMCs. The same amounts of the samples were loaded (10 ng of CA-p24 per lane). Briefly, a 30-μl sample was mixed with 6 μl (concentrated five times) of sodium dodecyl sulfate (SDS) sample buffer and was denatured by boiling for 3 min. The sample was separated on a SDS–10% polyacrylamide gel and was electrophoretically transferred to nitrocellulose. The immunoblot was stained with either a 1:1 mixture of the RT monoclonal antibodies 3019 and 716 or a pool of human HIV immunoglobulins (HIVIg) and was developed by the 5-bromo-4-chloro-3-indolylphosphate–nitroblue tetrazolium protocol (Sigma).

Exogenous and endogenous RT assays. The exogenous RT assay was used to measure the activity of RT enzymes extracted from virions or *E. coli*-produced RT enzymes. We analyzed [^{32}P]dTTP incorporation on the poly(rA) · oligo(dT) template-primer as described previously (2). The length distribution of the synthesized cDNAs was analyzed on denaturing urea-polyacrylamide sequencing gels. Samples of culture supernatants of infected SupT1 cells were used as the source of virions for the endogenous RT assay. This assay allows reverse transcription to proceed within particles from permeabilized virus and was performed as described by Schwartz et al. (43), with some minor deviations. A supernatant sample (450 μl; approximately 350 ng of CA-p24) was mixed with 50 μl of 10× TME buffer (500 mM Tris HCl [pH 8.0], 30 mM $MgCl_2$, 30 mM EDTA, 0.1% Nonidet P-40, and 100 or 1,000 μM dNTPs [25 or 250 μM, each, dNTP purified by high-performance liquid chromatography; Boehringer Mannheim]). The reaction mixtures (final concentration, 0.01% Nonidet P-40 and 10 or 100 μM dNTPs) were incubated for 2 h at 41°C, and the reaction was stopped by the addition of 25 μl of 10% SDS, 25 μl of 0.5 M EDTA, and 16.7 μl of 3 M NaCl. The virion samples were treated with proteinase K (final concentration, 100 μg/ml; Boehringer Mannheim) for 1 h at 56°C, and the synthesized cDNA was isolated by phenol-chloroform extraction. DNA products were precipitated with sodium acetate-ethanol and were dissolved in TE buffer (10 mM Tris HCl [pH 8.0], 1 mM EDTA). The samples were treated with NaOH (final concentration, 200 mM) to degrade the RNA template and to denature the cDNA products and were loaded directly onto 1% agarose gels. The Southern blot transfer was performed in 400 mM NaOH, and the blot was hybridized with ^{32}P-labeled probes directed against different regions of the HIV-1 genome. Two probes were used; a 712-bp *Pvu*II fragment of the R/U5 region of HIV-1 LAI (positions 433 to 1145) and a 2,190-bp *Pvu*II fragment of the Pol region (positions 1145 to 3335). The double-stranded DNA probes were labeled by the random labeling method (Boehringer Mannheim) and were denatured before use. Hybridization and subsequent washes were performed as described elsewhere (5). Hybridization signals were quantitated with a Phosphor Imager (Molecular Dynamics).

CA-p24 ELISA. CA-p24 levels were determined in culture supernatants by enzyme-linked immunosorbent assay (ELISA) (30, 32).

RESULTS

3TC-resistant RT enzymes extracted from virions produced by SupT1 cells show a functional defect. The 3TC-resistant HIV-1 variants 184Val and 184Ile demonstrate a replication defect that is restricted to PBMC cultures and that is not apparent in the transformed T-cell line SupT1. To test whether cell-type specific cofactors determine this difference, we performed in vitro reverse transcription assays with wild-type and 3TC-resistant RT enzymes that were extracted from virions produced by either cell type (SupT1 and PBMC in Fig. 1). In this poly(rA) · oligo(dT) assay, we included purified, recombinant forms of wild-type and 3TC-resistant RT protein (*E. coli* in Fig. 1). The SupT1-derived RT samples showed the typical activity and processivity pattern that we reported previously (Met [wild type] > Val > Ile [2]). A very similar activity and processivity pattern was observed for the set of RT enzymes isolated from virions from PBMCs and the recombinant RT forms, indicating that virion cofactors do not determine the differences in the replication potential of 3TC-resistant viruses in primary versus transformed cells. One cannot exclude the possibility that a SupT1 cofactor that is able to suppress the defect of 3TC-resistant RT is lost or inactivated during the RT extraction protocol. The results with the purified, recombinant RT samples indicate that mutation of residue 184 affects the intrinsic activity and processivity of the RT enzyme, consistent with previous studies with 184Val (7, 50).

It is formally possible that virions produced by PBMCs package less RT protein compared with virions produced by SupT1. If the RT concentration becomes limiting in the virions from PBMCs, the processivity defect may become more pronounced. We therefore analyzed the RT content of the two virion preparations. The RT enzyme activity was initially measured in supernatant samples of infected PBMC and SupT1 cultures. The RT enzyme activity was standardized for the amount of virions used, which was based on CA-p24 levels. These results indicated an approximately sixfold higher RT level in virions from SupT1 cells compared with those in virions from PBMCs (data not shown). However, culture supernatants of the two cell types may contain different amounts of free CA-p24 protein that is either secreted from cells or shed from virions. We therefore repeated this analysis with virions that were purified from the culture supernatant by ultracentrifugation (Table 1). The RT/CA-p24 ration was determined in triplicate with wild-type virus and the two 3TC-resistant variants. We measured an approximately threefold decrease in the RT/

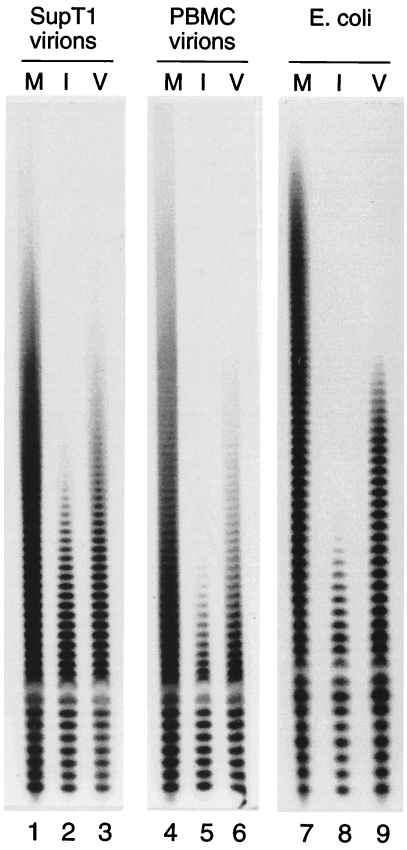

FIG. 1. Reduced activity of 3TC-resistant RT variants 184Val and 184Ile. The RT enzyme was extracted from virion particles produced by either the transformed T-cell line SupT1 or primary cells (PBMCs). In addition, the wild-type (M) and 3TC-resistant codon 184 variants 184Ile (I) and 184Val (V) were produced as recombinant fusion proteins in *E. coli*. All RT samples were assayed with the poly(rA) · oligo(dT) template-primer, and reaction products were analyzed on a 6.0% polyacrylamide–7.1 M urea sequencing gel.

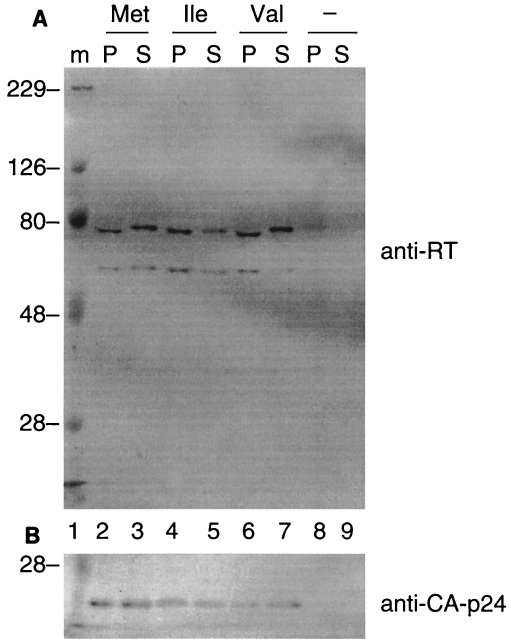

FIG. 2. Western blot analysis of RT protein from wild-type and mutant HIV-1 virions. HIV-1 virions were produced either in PBMC primary cells (P) or in the SupT1 T cell line (S). The same amounts of samples were loaded (10 ng of CA-p24 per lane). The blot was stained with either a mixture of RT monoclonal antibodies 3019 and 716 (A) or the HIVIg reagent that recognizes primarily CA-p24 protein (B). Lane m, molecular size marker proteins, with their position (in kilodaltons) indicated on the left; –, mock sample derived from the culture supernatant of uninfected PBMCs or SupT1 cells.

CA-p24 ratio for the virions from PBMC compared with that for virions from SupT1 cells.

One putative complication with the results presented above is that RT activity may not correlate directly with the amount of RT protein. In fact, it took much longer to produce sufficient virus in infected PBMC cultures than in infected SupT1 cultures. Consequently, the virions from PBMCs are on average older than the virions from SupT1 cells, and the virions from PBMC samples may have lost RT activity because of proteolysis or unfolding of the enzyme. To determine directly the amount of RT protein in virion particles, we performed semiquantitative Western blot analysis with the purified virion samples (Fig. 2). Equal amounts of virions, based on the CA-p24 levels as determined by ELISA, were loaded onto each lane (see also the gel in Fig. 2B, stained with polyclonal antibodies directed against CA-p24). The gel in Fig. 2A was probed with monoclonal antibodies against the RT protein. The results indicate that similar amounts of RT protein are packaged into virions produced by PBMCs and SupT1 cells (P and S in Fig. 2, lanes 2 and 3). Similar results were obtained in parallel experiments with the 184Ile (Fig. 2, lanes 4 and 5) and 184Val RT (Fig. 2, lanes 6 and 7) variants.

TABLE 1. Analysis of virions produced by PBMC and SupT1 cells

Virion source and mutant	RT activity (counts/μl [10^5])	CA-p24 concn (pg/μl)	RT/CA-p24 ratio (counts/pg)
PBMCs			
184Met	20.1	370	5,432
184Ile	5.9	370	1,595
184Val	7.5	300	2,500
SupT1 cells			
184Met	557	3,310	16,828
184Ile	174	4,310	4,037
184Val	195	2,550	7,647

The appearance of the two p66/p51 bands indicated that processing of the Pol precursor protein is efficient in both cell types. A minor difference in migration is apparent for the p66/p51 bands in the PBMC samples compared with that in the SupT1 samples, but we think that this shift in migration is an artifact caused by the presence of an abundant protein of approximately 70 kDa in the PBMC samples (visual inspection of the unstained blot did reveal this protein of unknown identity). To prove that the p66/p51 subunits of both cell types are identical in size, we mixed the two samples before SDS-gel electrophoresis; this did abolish the migration difference (data not shown). Similar results were obtained for the two 3TC-resistant virions, indicating that both the wild-type and mutant Pol polyproteins are processed correctly to the mature p66/p51 heterodimer. It is also obvious that there is no significant proteolytic degradation of the mutant proteins, as has been reported for other drug-resistant RT mutants (35).

Manipulation of the intracellular dNTP pool in infected cells. Replication experiments with SupT1 and PBMCs were performed in the presence of increasing amounts of HU. HU

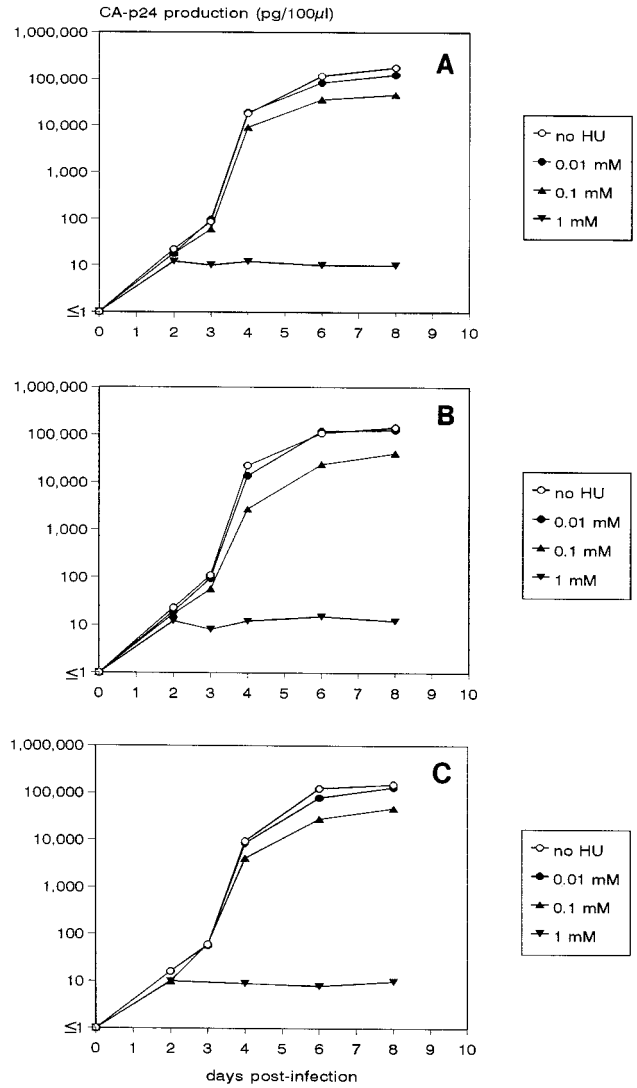

FIG. 3. Effect of HU on the replication of 3TC-resistant HIV-1 variants in SupT1 cells. SupT1 cells were infected with the three HIV-1 variants (inoculum of 50 ng of CA-p24) and were cultured in the absence or presence of increasing amounts of HU (0.01, 0.1, and 1 mM). CA-p24 antigen levels were measured in samples taken on days 0, 2, 3, 4, 6, and 8. Severe toxicity was apparent by visual inspection of the cells at the 1-mM HU concentration. (A) Met variant; (B) Ile variant; (C) Val variant.

is an inhibitor of the ribonucleotide reductase enzyme that catalyzes the reduction of ribonucleotides to deoxyribonucleotides. HU treatment leads to a reduction of the cellular dNTP pool. First, we compared the replication capacity of wild-type and 3TC-resistant HIV-1 in SupT1 cells (Fig. 3). This cell type and other transformed T-cell lines were reported to contain high levels of intracellular dNTPs (2, 31). HIV-1 replication in SupT1 cells is apparently not very sensitive to reduction of the dNTP pool, because virus production was not inhibited by HU treatment. In particular, no selective defect of the 3TC-resistant variants was apparent in these experiments. The toxic effects of HU were minimal at concentrations of ≤0.1 mM, similar to the findings of previous studies (17, 26). Higher concentrations (1 mM) led to cell death and, consequently, cannot be used to study virus replication. Such high HU levels prolong the S phase of the cells by dNTP depletion, and prolonged incubation leads to apoptosis (52).

We next tested the effect of the drug HU on HIV-1 replication in PBMCs. These primary cells have a 20-fold lower dNTP concentration than those in SupT1 cells (2) and are expected to be more sensitive to further suppression of the dNTP level. Consistent with previous reports on HU treatment (16, 25, 26), we measured a small reduction in the rate of replication of the wild-type virus at 0.1-mM HU (Fig. 4). Nearly complete inhibition of replication was observed for the 184Ile variant under these conditions. Because the differences in this infection assay are relatively small, in particular for the 184Val variant, we performed a similar replication experiment with PBMCs that were transfected with the three proviral DNA constructs (Fig. 5). Partial inhibition of 184Val and complete inhibition of 184Ile were observed. These combined results suggest that the 3TC-resistant variants are sensitive to reduction of the intracellular dNTP pool in primary cells. The observed rank order of HU sensitivity (Ile > Val > Met) is

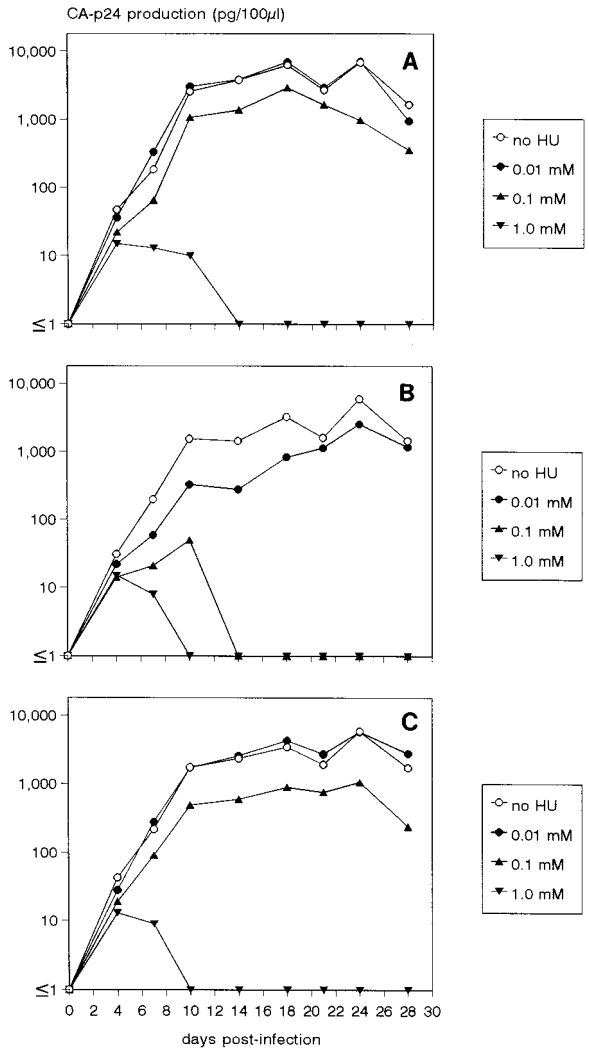

FIG. 4. Effect of HU on the replication of 3TC-resistant HIV-1 variants in infected primary cells. PBMCs were activated by with phytohemagglutinin and infected with the wild-type or 3TC-resistant HIV-1 viruses (inoculum of 30 ng of CA-p24). Cells were cultured in the absence or presence of HU (see legend to Fig. 3 for details). CA-p24 antigen levels were measured in the culture supernatant twice a week for 4 weeks. Visual inspection of the PBMC culture indicated toxicity at the 1-mM HU concentration. (A) Met variant; (B) Ile variant; (C) Val variant.

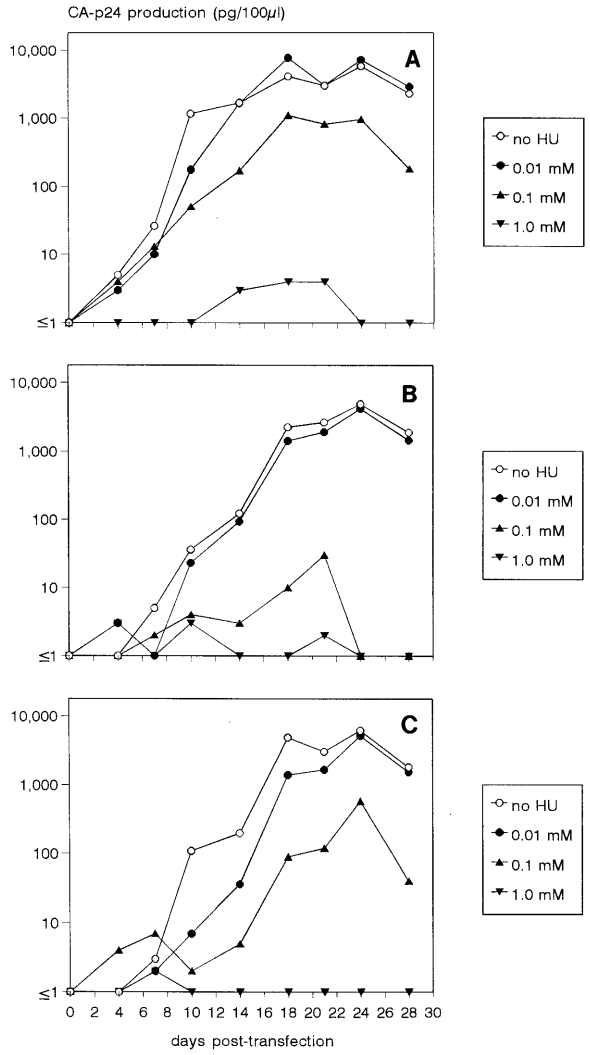

FIG. 5. Effect of HU on the replication of 3TC-resistant HIV-1 variants in transfected primary cells. PBMCs were stimulated with phytohemagglutinin and transfected with 10 μg of the wild-type or mutant molecular clones. Cells were cultured in the absence or presence of HU (see legend to Fig. 3). CA-p24 antigen levels were measured in the culture supernatant twice a week for 4 weeks. (A) Met variant; (B) Ile variant; (C) Val variant.

consistent with the magnitude of the processivity defect measured for these RT variants.

Reverse transcription in permeabilized virions at different dNTP levels. The dNTP level can be accurately controlled in cell-free reverse transcription assays. However, this reaction may not accurately mimic the reverse transcription reaction that takes place within partially uncoated virion particles in the cytoplasm of infected cells. Manipulation of the intracellular dNTP pool is possible to some extent, but drugs such as HU also affect other cellular processes and are toxic at high concentrations (see above). Alternatively, reverse transcription can be studied in virions upon permeabilization of the lipid bilayer with detergent to allow entry of exogenously added dNTPs (1, 43, 47, 54). This endogenous reverse transcription may reliably mimic the native reverse transcription process and allows one to control dNTP levels.

The wild-type and 3TC-resistant virions were treated with the detergent Nonidet P-40 and were incubated for 2 h at 41°C in a buffer containing 10 or 100 μM dNTPs. The cDNA products of reverse transcription were extracted from the virions and analyzed on Southern blots (Fig. 6). To visualize the early reverse transcription products, we used an R/U5 region probe that detects the initial strong-stop cDNA and all longer products (Fig. 6A). A control probe directed against the Pol region was used to selectively detect extended cDNA products (Fig. 6B). The cDNA length profile was analyzed by Phophor Imager scanning of individual lanes of the Southern blot. The results are shown for the wild-type (184Met) and the 3TC-resistant variant (184Ile) at low and high dNTP levels (Fig. 7). Reverse transcription is apparently restricted at the 10 μM dNTP level because strong-stop cDNA was made inefficiently and larger cDNA products were not observed (Fig. 6A and B, lanes 1 to 3). Significant levels of extended cDNAs were produced in virions treated with 100 μM dNTP (Fig. 6A and B, lanes 5 to 7). Most importantly, a significant difference in the amount of strong-stop cDNA synthesis was measured for the wild-type and 3TC-resistant RT enzymes at the suboptimal level of 10 μM dNTP (Fig. 6A, lanes 1 to 3). Quantitation of the strong-stop cDNA band revealed that the Val variant had 53% of the activity of the wild-type enzyme and that the Ile variant was only 45% as active as the wild-type enzyme. This activity pattern (Met [wild-type] > Val > Ile) mirrors the activity spectrum of the purified RT enzymes in the poly(rA) · oligo(dT) assay (Fig. 1). Interestingly, no reduced RT activity of the Val and Ile enzymes was apparent in virions treated with 100 μM dNTPs (Fig. 6A and B lanes 5 to 7).

DISCUSSION

The RT protein was isolated from virions produced by primary and transformed cell types to detect possible differences in enzyme activity that may be caused by cell-type specific cofactors. No difference in the amount of RT per virion was measured, and the typical activity and processivity profile of the wild-type and 3TC-resistant RT variants was similar for virons produced by the two cell types. We subsequently demonstrated that cell-type differences in the activities of the 3TC-resistant variants is determined by the intracellular dNTP level.

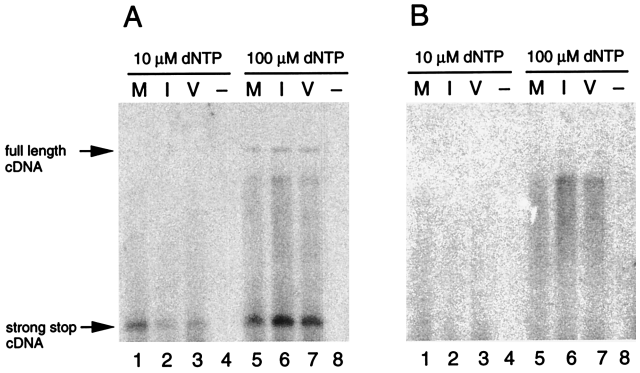

FIG. 6. Endogenous reverse transcription reaction with wild-type and 3TC-resistant virions. The wild type (M) and variants (I and V) were produced by SupT1 cells. These samples and a mock culture supernatant (marked with a minus sign) were treated with 0.01% Nonidet P-40 to permeabilize the virion membrane for uptake of added dNTPs. Samples were incubated for 2 h at 41°C in the presence of exogenously added dNTPs (lanes 1 to 4, 10 μM; lanes 5 to 8, 100 μM). The DNA was extracted by phenol treatment and was subsequently separated on a 1% agarose gel and blotted onto a nylon membrane. This blot was incubated with a R/U5 region probe that detects early cDNA products (e.g., strong-stop cDNA) (A) or with a Pol probe that detects more extended cDNAs (B). The positions of strong-stop and full-length cDNA products are indicated on the left of panel A.

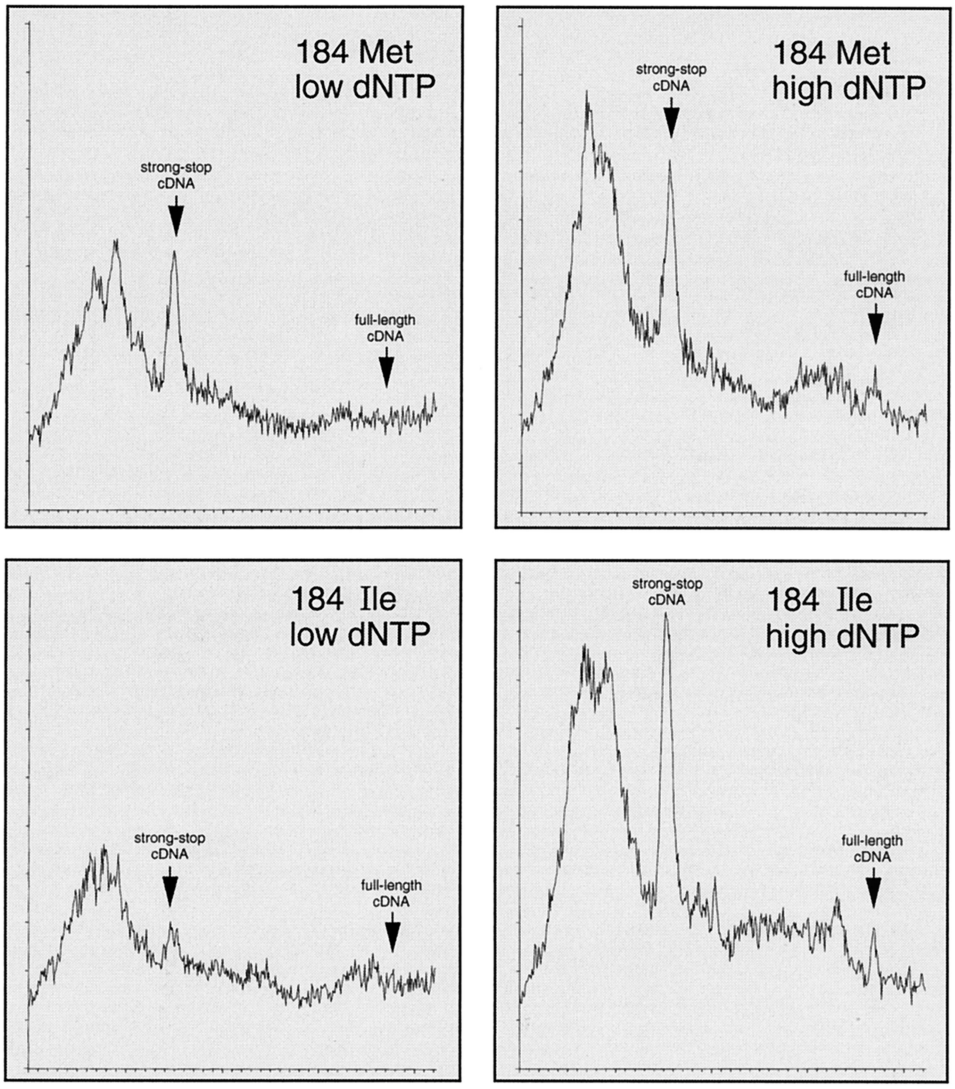

FIG. 7. cDNA synthesis profile of the wild-type (Met) and 184Ile mutant virions at low and high dNTP levels. The samples from Fig. 6A, lanes 1, 2, 5, and 6, were quantitated with a Phosphor Imager (Molecular Dynamics). The positions of the strong-stop and full-length cDNA products are indicated.

First, the dNTP level was manipulated in infected cells by the drug HU. Second, the endogenous reverse transcription assay was performed with permeabilized virions in the presence of various amounts of dNTPs. The combined results document that the enzyme defect of the 3TC-resistant enzymes is predominant at limiting dNTP levels. This sensitivity to the dNTP concentration may be a rather specific property of the codon 184 RT mutants that provide 3TC resistance, but primary cell types are recommended for use in fitness and functional studies with other drug-resistant RT variants.

Cell-type-specific effects on HIV-1 replication have been reported in several systems. For instance, some of the HIV-1 accessory gene products contribute to virus replication in a cell-type-dependent manner (for a review, see reference 27). It has also been known for some time that the process of reverse transcription is particularly sensitive to the type of host cell, which has been demonstrated to reflect differences in the intracellular dNTP pool (11, 31, 34, 36, 53). However, the differential behavior of drug-resistant RT variants in particular cell types is rather uncommon. One other study reported a similar cell type-specific defect for RT variants resistant to the class of BHAP drugs (35). The extent of this cell type difference in RT function is rather dramatic for the codon 184 RT variants. For instance, we recently obtained a novel 3TC-resistant variant (184Thr) by a limiting dilution selection protocol in SupT1 cell cultures (22). This variant RT enzyme is poorly active [activity of approximately 12% of that of the wild-type enzyme in the poly(rA) · oligo(dT) assay], and the corresponding virus replicates inefficiently in primary cells. Despite this dramatic enzyme defect, normal replication kinetics were measured in transformed SupT1 T cells (22).

Restriction of host cell dNTP synthesis has been proposed as a general strategy for inhibiting the rate of HIV-1 replication. For instance, the drug HU reduces the intracellular dNTP concentration and has been demonstrated to inhibit HIV-1 DNA synthesis and viral replication (16, 25, 26). However, it has also been suggested that interference is observed only at HU doses that inhibit cell proliferation (17; this study). Consistent with these results, monotherapy with relatively low HU levels in HIV-infected individuals did not provide a benefit

(18). At least for monotherapy, the concentration of drug required to affect HIV replication by depleting cellular dNTP pools is too close to or even above the concentration that causes cell toxicity during prolonged treatment.

Alternatively, there is some evidence that HU may act synergistically in combination with other RT drugs (15, 17, 26, 28). We proposed a specific use of HU in combination with 3TC on the basis of the observation that the 3TC-resistant RT enzymes 184Val and 184Ile are particularly sensitive to lowering of the dNTP pool (2). Reduced processivity was measured for these 3TC-resistant RT enzymes, and this may explain their hypersensitivity to dNTP depletion compared with the sensitivity of the wild-type enzyme. It was reasoned that the fitness of 3TC-resistant viruses may decline further at low dNTP conditions induced by HU. In this study, we demonstrate that the fitness of 3TC-resistant viruses is selectively repressed at low intracellular dNTP concentrations. Thus, combination therapy with 3TC and a second drug that reduces the intracellular dNTP pool may be particularly powerful. We demonstrate such a sensitizing effect with HU, but a significant effect was measured only in primary cells in the presence of high HU levels that are near those that induce cell death. It is therefore doubtful whether this specific combination will be useful clinically. However, other drugs that influence the dNTP pool, e.g., 2′-substituted deoxycytidine analogs (17), may have certain advantages over HU.

The 184Val RT enzyme demonstrates reduced activity and processivity. Interestingly, it has also been proposed that 184Val exhibits improved fidelity with respect to nucleotide misincorporation (3, 37, 48). It has been reported that this effect incapacitates the ability of HIV-1 to acquire additional resistance-conferring mutations, and the concept of sequential therapy with 3TC as the first drug has been proposed (48). We measured an even larger increase in the fidelity of the 184Ile variant in cell-free assays (36a), but the in vivo relevance of this change in enzyme fidelity is not clear. For instance, there is accumulating evidence that 3TC-resistant viruses can rapidly accumulate other drug resistance mutations in tissue culture (4, 23). We notice that biochemical fidelity assays are performed with limiting dNTP levels, whereas most tissue culture infections are performed with transformed T-cell lines that have a large dNTP pool. It therefore remains possible that significant fidelity differences are manifest in primary cell types because of a lower dNTP pool. However, recent clinical evidence documents the rapid appearance of double-resistant virus in patients treated initially with 3TC and subsequently with a combination of 3TC and AZT (23); see also the references in reference 23).

ACKNOWLEDGMENTS

We thank Jeroen van Wamel for technical support and Belinda B. Oude Essink for help in the construction of recombinant RT vectors. Monoclonal antibodies 3019 and 716 and HIVIg were kindly provided by A. Prince, D. Hellund, A. M. Szilvay, and P. Yoshihara and were obtained through the NIH and MRC AIDS Repositories.

This project was sponsored in part by the Dutch AIDS Foundation (AIDS Fonds).

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