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Reduced replication of 3TC-resistant HIV-1 variants in primary cells due to a processivity defect of the reverse transcriptase enzyme

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Human immunodeficiency virus type 1 (HIV-1) variants with resistance mutations in the reverse transcriptase (RT) gene appear during drug therapy with the nucleoside analogue 2′,3′-dideoxy-3′-thiacytidine (3TC). These resistance mutations alter the methionine (Met) residue of the conserved YMDD motif, which is part of the catalytic core of the RT enzyme. Isoleucine (Ile) variants are initially observed, followed by the appearance and eventual outgrowth of viruses encoding valine (Val). Similar replication kinetics were measured for wild-type and 3TC-resistant HIV-1 viruses in tissue culture infections of a T cell line, but we measured reduced polymerase activity for the two mutant RT enzymes compared with the wild-type enzyme (Ile = 43% and Val = 67%). Gel analysis of the reverse transcription products revealed that both 3TC-resistant RT mutants produce significantly shorter cDNA molecules than the wild-type enzyme [Met (wt)>Val>Ile], indicating that 3TC-resistant RT polymerases are less processive enzymes. Interestingly, these enzyme defects were more pronounced under limiting dNTP concentrations and we therefore assayed virus replication in primary cells that contain relatively low dNTP levels. Under these conditions, we measured significantly reduced replication kinetics for the 3TC-resistant HIV-1 variants [Met (wt)>Val>Ile]. If the level of virus replication can be similarly reduced in 3TC-treated patients that develop drug-resistant HIV-1 variants, this may be of considerable clinical benefit.

Keywords: drug resistance/enzyme processivity/virus fitness

Introduction

The reverse transcriptase (RT) enzyme, which is an RNA- and DNA-dependent DNA polymerase, is an excellent target for inhibition of the multiplication of the human immunodeficiency virus type 1 (HIV-1). Besides being an essential enzyme in the viral replication cycle, its properties are quite different from those of the cellular DNA polymerases. Nucleoside analogues, particularly those belonging to the dideoxynucleoside family, can inhibit the RT enzyme. 3′-Azido-2′,3′-dideoxythymidine (AZT) was the first drug tested in HIV-infected individuals, and other drugs like ddI and ddC also belong to this class of 'chain terminators'. These drugs act as terminators during reverse transcription by preventing the formation of 3′,5′-phosphodiester bonds. More recently, a new generation of dideoxynucleosides (e.g. 3TC and D4T) has been developed and other chemically diverse anti-RT drugs were identified (e.g. benzodiazepine analogues like TIBO). However, resistance to all these compounds develops rapidly, and this appears to blunt their clinical use (Larder and Kemp, 1989; Erice and Balfour, 1994; Larder, 1994). HIV-1 drug resistance has been associated with distinct mutations in the RT enzyme.

The virtually inevitable and rapid occurrence of mutations causing resistance to therapeutic agents has strong implications for clinical applications. The prospect is not completely hopeless, however, because there may be drugs (or drug combinations) that do not allow resistance to develop. Furthermore, drug-resistant RT proteins may exhibit decreased enzyme function and lead to a reduction in viral fitness, and perhaps this can be facilitated by multiple mutations selected by a combination of RT inhibitors. Based on new data on the kinetics of virus replication *in vivo* (Ho *et al.*, 1995; Wei *et al.*, 1995), it has been speculated that a 2-fold reduction in the number of productively infected cells might lead to a 2-fold increase in the mean clinical latency (Coffin, 1995). Thus, it seems possible that even a partially reduced level of virus replication is of considerable clinical benefit, although the clinical outcome will depend to a large extent on the currently unknown relationship between virus replication levels and AIDS pathogenesis.

In this study, we analysed the RT enzyme function and viral replication characteristics of HIV-1 variants resistant to the nucleoside analogue 3TC, the (–) enantiomer of 2′,3′-dideoxy-3′-thiacytidine. This drug potently inhibits HIV-1 replication *in vitro* (Coates *et al.*, 1992a,b; Schinazi *et al.*, 1992) and *in vivo* (van Leeuwen *et al.*, 1995; Schuurman *et al.*, 1995). 3TC-resistant virus variants with an ~1000-fold reduced sensitivity are selected in the presence of 3TC both *in vivo* and in cell culture (Boucher *et al.*, 1993; Gao *et al.*, 1993a; Schinazi *et al.*, 1993; Tisdale *et al.*, 1993; Schuurman *et al.*, 1995). The wild-type methionine (Met) at amino acid position 184 in the catalytic site of the RT enzyme was consistently found to be replaced by either valine (Val) or isoleucine (Ile) (Boucher *et al.*, 1993; Gao *et al.*, 1993a; Tisdale *et al.*, 1993). These changes were confirmed to be both required and sufficient for high level resistance *in vitro* (Boucher *et al.*, 1993; Gao *et al.*, 1993a; Tisdale *et al.*, 1993). Because this resistance mutation is located within the well-conserved nucleotide binding pocket of the HIV-1 RT

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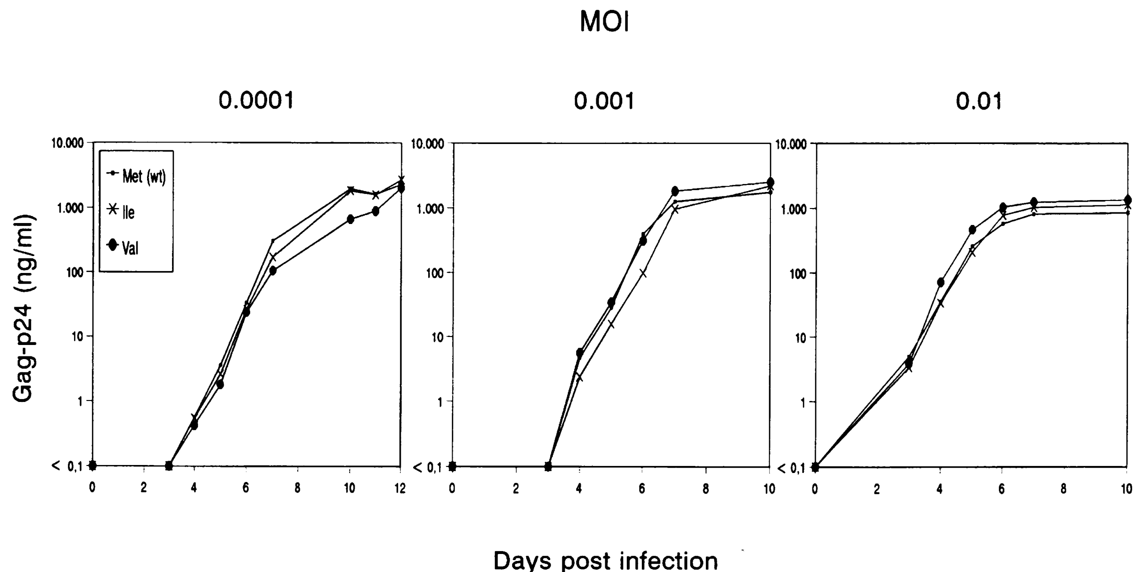

Fig. 1. Efficient replication of 3TC-resistant HIV-1 variants in the SupT1 T cell line. The SupT1 cell line was infected with the three 184 variant HIV-1 viruses at different multiplicities of infection [m.o.i. = 0.0001 (left), 0.001 (middle) or 0.01 (right)]. Virus production was monitored by measuring HIV-1 CA-p24 antigen levels in the culture supernatant (Moore *et al.*, 1990; McKeating *et al.*, 1991).

protein (Kamer and Argos, 1984; Inokuchi and Hirashima, 1987; Argos, 1988; Larder *et al.*, 1989; Poch *et al.*, 1989; Jablonski *et al.*, 1991; Le Grice *et al.*, 1991), its enzymatic properties may have been affected. In this study, we demonstrate reduced processivity of the mutant RT enzymes and reduced replication levels of the 3TC-resistant viruses in primary lymphoid cells.

Results

Reduced enzyme activity of 3TC-resistant reverse transcriptase

Mutant RT genes encoding the Met184Ile or Met184Val change (codon change ATG to either ATA or GTG, respectively) were generated by site-directed mutagenesis in the HXB2 molecular clone of the HIV-1 virus. Virus stocks were prepared and used to infect the SupT1 T cell line at different multiplicities of infection (m.o.i. 10^{-4}, 10^{-3} and 10^{-2}). Samples of the culture supernatant were taken at several days post-infection and assayed for CA-p24 production (Figure 1), and the infectious titre was determined by plaque-forming units on HeLa-CD4$^+$ cells. No difference was measured in viral replication kinetics (Figure 1) and virus titres for wild-type and 3TC-resistant viruses (data not shown). The genomes of the two 184 variants were sequenced after prolonged culture (± 12 weeks) in order to exclude reversion of the codon 184 mutations. Both codon 184 variations were found to be stably maintained, which was confirmed by the 3TC-resistant phenotype of the cultured virus (data not shown).

Virus samples were used to assay for polymerase activity of the wild-type and mutant RT enzymes. The RT protein was released from the virions by NP-40 treatment (0.5% final concentration), and equal amounts of protein, based on CA-p24 levels, were assayed for RT activity in a poly(rA)·oligo(dT) assay. The activity obtained for the wild-type RT enzyme (184Met) was set at 100% for each individual experiment. The RT activity of the two mutant enzymes was consistently lower than wild-type RT in 10 independent assays (summarized in Table I). Compared with the wild-type RT enzyme, the Val variant exhibited 67% activity and the Ile variant was only 43% active. Next, we assayed the enzymatic properties of the three virion-extracted enzymes at different reaction temperatures (Figure 2). The activity of all three RT polymerases increased as a function of temperature, with optima between 30 and 42°C. A sharp decline in enzyme activity was observed at higher temperatures. Most importantly, the 184Val and 184Ile enzymes exhibited significantly reduced activity at all assay temperatures when compared with the wild-type 184Met enzyme.

Since the observed differences in activity of the 3TC-resistant RT variants are relatively small, we wanted to verify this effect with an additional set of mutant HIV-1 viruses. We therefore introduced the 184 mutations into an AZT-resistant RT gene isolated from an AZT-treated patient. This RT gene contains two mutations that confer AZT resistance (amino acid substitutions M41L and T215Y). We measured no difference in virus replication rate for the 184 variants compared with wild-type in this AZT-resistant background (data not shown). Nevertheless, the virion-derived RT enzymes displayed distinct differences in polymerase function (Table I), and the activity spectrum [Met (wt)>Val>Ile] was consistent with that observed for the original set of 184 mutants.

To compare directly the amount of RT enzyme present in the virion lysates, we performed a Western blot assay on these samples (Figure 3). This experiment indicates that similar amounts of RT protein are released from wild-type and variant viruses. Furthermore, all three RT proteins were present in the mature p66/p51 form. Reduced activity of the mutant RT enzymes could also be due to decreased protein stability. To test this, the virion-extracted RT enzymes were pre-incubated either for a variable time period (0–16 h) or at different temperatures (4–46°C), followed by a standard poly(rA)·oligo(dT) activity assay. We plotted the remaining RT activity for each individual RT enzyme (Figure 4A and B, respectively). No difference

Table I. Enzymatic properties of the 184 RT variants

	HXB2 (recombinant virus)		HXB2 (molecular clone)		Patient (recombinant virus; AZT-resistant)	
	RT activity[a,b]	Processivity[c]	RT activity[b]	Processivity[c]	RT activity[b]	Processivity[c]
Met (wt)	100%	80 nt	100%	60 nt	100%	64 nt
Ile	43 ± 7%	50 nt	53%	47 nt	28%	54 nt
Val	67 ± 9%	70 nt	82%	58 nt	35%	60 nt

[a]Average of 10 independent assays.
[b]Relative polymerase activity on poly(rA)·oligo(dT) (wild-type activity was set at 100%).
[c]Maximal processivity value (in nucleotides) on poly(rA)·oligo(dT).

in enzyme stability was measured; e.g. all three RT proteins were stable at 30°C for up to 3 h and we measured similar half-lives of ~1 h at 37°C.

Reduced processivity of 3TC-resistant RT enzymes

To obtain a more detailed understanding of the reduced polymerase activity of the HIV-1 RT variants, we conducted processivity analysis of the HIV-1 RT variants. Processivity of a polymerase is defined as the number of nucleotides incorporated before the enzyme dissociates from the template (Majumbar *et al.*, 1988; Huber *et al.*, 1989). The length distribution of the cDNAs synthesized with a poly(rA)·oligo(dT) template–primer complex was analysed on a denaturing polyacrylamide gel (Figure 5). Corresponding RT activities, as measured in the filter assay, are indicated on the right of the individual lanes. Consistent with the data shown above, we measured reduced RT activity for both 184 mutants. An analysis of the length distribution of the cDNAs synthesized by the wild-type and mutant RT enzymes revealed that reduced RT activity correlates with the synthesis of shorter cDNA products. The results of 10 different processivity experiments are summarized in Table I. The wild-type RT enzyme produced poly(dT) molecules with a maximal length of 80 nucleotides, whereas the 184Val and Ile variants were only processive for up to 70 and 50 nucleotides, respectively. Similar results were obtained with the second set of 184 mutants in the context of an AZT-resistant RT gene (Table I).

Several control experiments were performed. Processivity should be measured under conditions that include great template excess and limiting polymerase, such that each synthesized cDNA molecule results from a single event of processive synthesis. We calculated that RT enzyme was present in a 1:50 molar ratio to the primer–template complex and that only a minority of the primer–templates was extended. Template excess was verified by conducting the measurement over a series of RT concentrations and by showing that, as the level of RT enzyme was decreased, the number but not the length of the cDNA products was reduced (Figure 5A). Processivity differences are also apparent by comparing the reactions with 10 µl of the Ile enzyme and 5 µl of the wild-type RT (Figure 5A). These samples produce approximately equal amounts of labelled cDNA based on the filter assay ($12\,423\times10^3$ and $12\,346\times10^3$ counts, respectively), but gel analysis indicates a significant difference in size distribution of these cDNAs.

As an additional control, we performed a template–primer challenge experiment. A 50-fold molar excess of the poly(rC)·oligo(dG) hybrid was added to a pre-formed RT–poly(rA)·oligo(dT) complex before addition of dTTP. This ensures that RT dissociating from the extended dT primer will be trapped by the excess poly(rC)·oligo(dG). Addition of the trap did not change the activity and

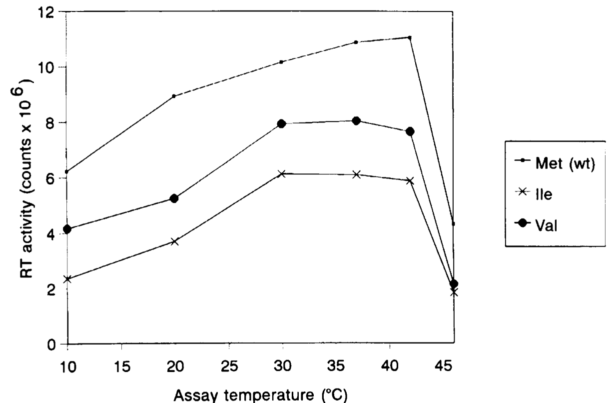

Fig. 2. Polymerase activity of the 3TC-resistant RT mutants at different temperatures. RT assays were performed on the poly(rA)·oligo(dT) template–primer at the indicated temperatures. RT activity is presented as arbitrary PhosphorImager counts.

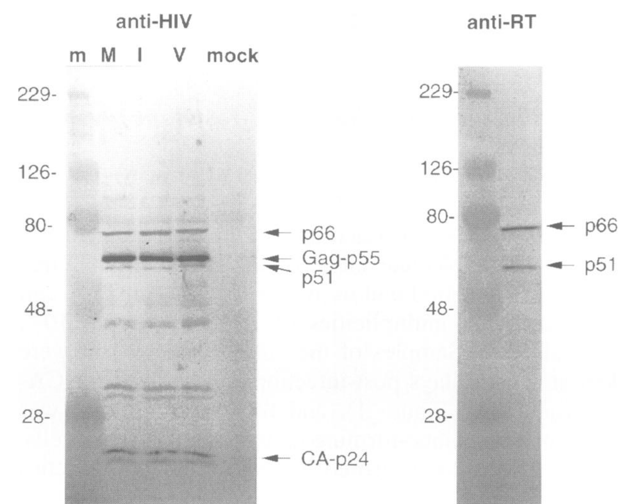

Fig. 3. Western blot analysis of RT protein from wild-type and mutant HIV-1 virions. Immunoblot analysis of purified HIV-1 virions. Equal amounts of the samples were loaded based on the CA-p24 levels (85 ng/lane). The blot was stained either with a pool of HIV-1 human immunoglobulins (HIVIG, left) or with a 1:1 mixture of RT monoclonal antibodies (right). Lane m contains molecular weight marker proteins, with their position (in kDa) indicated on the left. M, I and V represent the 184 Met, Ile and Val RT variants; mock is a sample derived from mock-transfected cells. The position of Gag proteins (Gag-p55 and CA p24) and both RT subunits (p66 and p51) is indicated on the right.

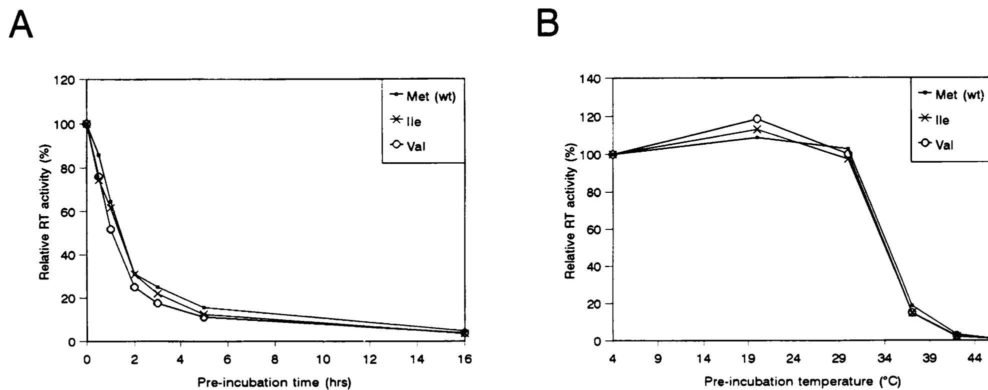

Fig. 4. Stability of the wild-type and mutant RT enzymes. RT samples were incubated either at 37°C for varying times (**A**) or for 3 h at the indicated temperatures (**B**). The RT activity was measured subsequently in the standard poly(rA)·oligo(dT) assay. Plotted is the remaining RT activity, with the activity of untreated RT samples (kept on ice during the pre-incubation) set at 100%.

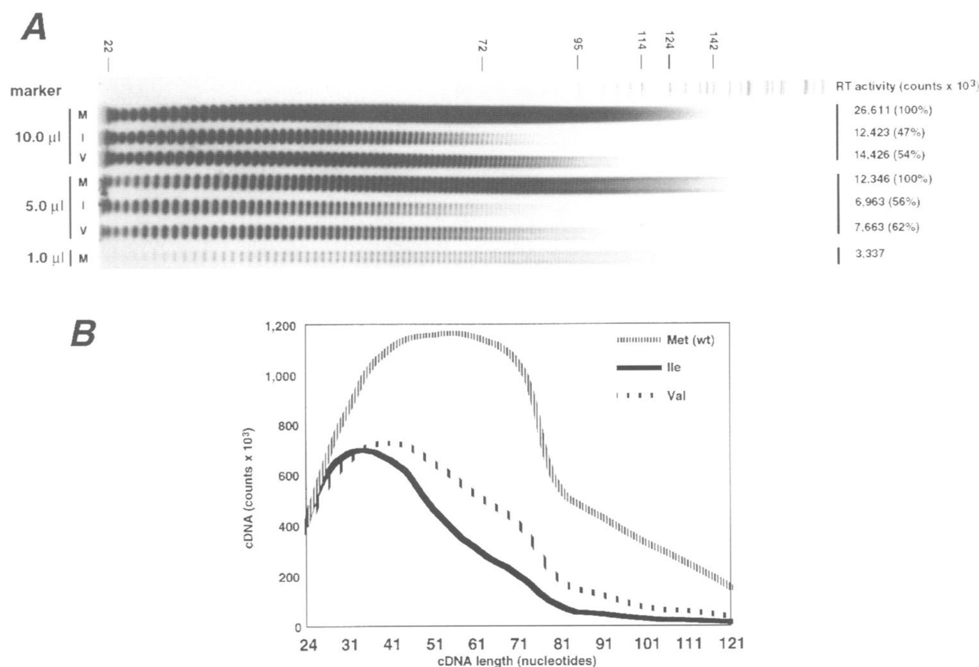

Fig. 5. 3TC-resistant RT enzymes are less processive on poly(rA)·oligo(dT). Wild-type and variant RT enzymes were extracted from virions, corrected for CA-p24 levels and assayed in the poly(rA)·oligo(dT) reaction. Reaction products were analysed on gel (**A**) and quantitated for total [^{32}P]dTTP incorporation. These absolute activities are shown on the right (the relative activity is shown within brackets, with the activity obtained for wild-type RT set at 100%). For gel analysis, reaction samples were ethanol precipitated and solubilized in formamide sample buffer. The length distribution of the cDNA products was analysed on a 6.0% polyacrylamide–7.1 M urea sequencing gel. A dideoxy sequencing reaction on an M13 DNA template with the M13/pUC reverse sequencing primer (T track only) was used as size marker. (**B**) The size distribution of cDNAs synthesized by wild-type and 184 variant RT. We quantitated the intensities of the cDNA bands in individual lanes of the polyacrylamide gel with a PhosphorImager.

processivity of the three RT enzymes (Figure 6A and B), indicating that our polymerase assays do reflect a single cycle of processive reverse transcription.

Since multiple primers can anneal to the homopolymeric template in the poly(rA)·oligo(dT) assay, it is formally possible that reduced activity of the 184 variants results from altered strand displacement properties. However, we calculated that, on average, one primer was annealed to every 500 nucleotides of the poly(rA) template, thereby excluding this possibility.

To test whether the observed differences in cDNA product lengths reflect a general property of the 184-mutated RT enzymes, we performed polymerase assays with other RNA homopolymeric templates. We measured more reduced RT activity with these primer–template combinations than for poly(rA)·oligo(dT). This biased template usage [poly(rA)·oligo(dT)>>poly(rC)·oligo(dG)>poly(rI)·oligo(dC)>poly(rU)·oligo(dA)] was observed previously with other polymerase enzymes (Baltimore and Smoler, 1971). Processivity analyses with poly(rI)·oligo (dC) demonstrated the typical Met (wt)>Val>Ile pattern, but no difference in cDNA length was observed with poly(rC)·oligo(dG) (data not shown). No cDNA synthesis was measured with the poly(rU)·oligo(dA) template–primer pair (data not shown). These results may suggest that the processivity properties of the HIV-1 RT enzyme are dependent on the sequence of the nucleic acid template. Similar sequence context effects were reported previously

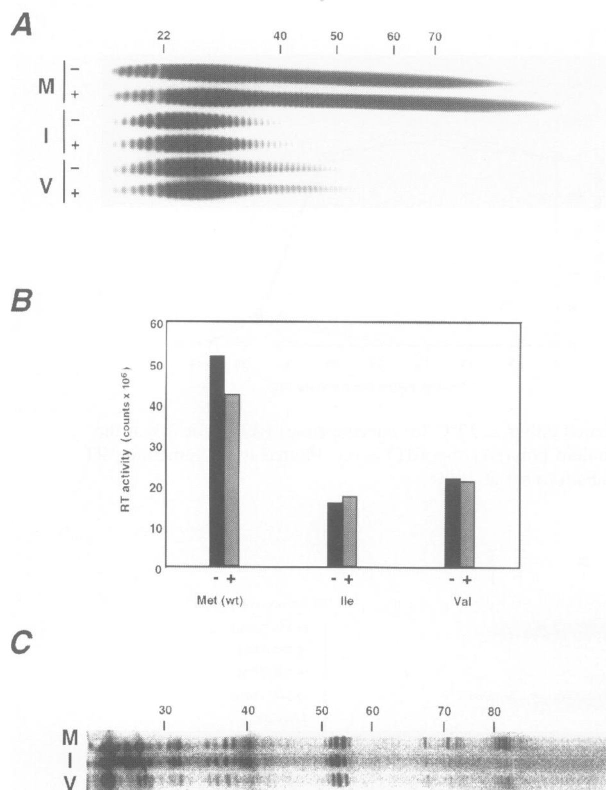

Fig. 6. Polymerization on poly(rA)·oligo(dT) in the presence of trap and on a heteropolymeric template. A 50-fold molar excess of the poly(rC)·oligo(dG) hybrid was added to a pre-incubated RT–poly(rA)·oligo(dT) mixture, followed by addition of radiolabelled dTTP. This ensures that RT dissociating from the extended primer will be trapped by the excess poly(rC)·oligo(dG) (cold trap). We analysed the products of the RT assay in a denaturing gel (**A**) and on a DE81 filter (DEAE ion-exchange paper) that was quantitated with a PhosphorImager (**B**). The heteropolymeric template is M13ss DNA and reverse transcription was initiated from an oligonucleotide primer (**C**).

for processivity and fidelity properties of several polymerases including the RT enzyme (Goodman *et al.*, 1993). We also tested the three RT enzymes on a heteropolymeric DNA template (Figure 6C). The processivity is limited in this system by polymerase stalling at specific pause/stop sites along the template, but the enzymes terminated with different efficiencies at these preferred stop sites. Consistent with the defects observed on some homopolymeric templates, we measured larger cDNA products for wild-type RT compared with the 184 mutants (Met> Val>Ile).

To ask whether these activity/processivity defects are specific for virion-extracted RT enzyme, we produced all three RT forms as recombinant protein in *Escherichia coli*. Based on poly(rA)·oligo(dT) assays, we found the activity spectrum of the recombinant enzymes to be indistinguishable from virion-derived RT (B.B.Oude Essink *et al.*, our unpublished data). Similar differences in activity and processivity of *E.coli*-expressed RT enzymes with mutations at residue 184 were reported previously (Wakefield *et al.*, 1992; Boyer and Hughes, 1995). This result also indicates that no cellular and/or viral co-factor is responsible for the reduced processivity of the 184Val and 184Ile enzymes (see Discussion).

Pronounced defects in RT processivity at low $MgCl_2$ and dNTP concentrations

The aspartic acids of the YMDD motif have been postulated to be involved in metal ion coordination at the catalytic active site of the RT enzyme. Thus, substitution of the Met184 residue may affect the metal ion requirements of the RT enzyme. To test this hypothesis, the *in vitro* poly(rA)·oligo(dT) assay was done under varying ionic conditions. First, we investigated the impact of varying the concentration of $MgCl_2$ (0–10 mM). In these reactions with virion-extracted RT enzyme, maximal processivity was obtained at concentrations ⩾5 mM Mg^{2+} for all three RT variants (Figure 7). The maximal number of nucleotides incorporated under these conditions is 94, 46 and 28 nucleotides for the Met, Val and Ile enzymes, respectively. At sub-optimal concentrations of 1 mM Mg^{2+}, these values are 24, six and three nucleotides, respectively. Second, we assayed the enzymatic activity in the presence of Mn^{2+} as divalent metal ion. All three RT enzymes demonstrated much reduced activity with Mn^{2+} as metal ion (~2% of the activity obtained in a standard assay with Mg^{2+}, data not shown). However, the relative RT activity of the 184 mutants was very similar to that observed in the standard polymerase assay (Val = 61%, and Ile = 37%).

RT processivity was reported to be sensitive to a variety of other factors, including template sequence/structure, dNTP levels and assay temperature (Bebenek *et al.*, 1989; Huber *et al.*, 1989; Dudding *et al.*, 1991; Klarmann *et al.*, 1993). We therefore tested the processivity of the RT mutants in the poly(rA)·oligo(dT) assay with different dTTP concentrations. The first observation is that the dTTP level in the standard assay is limiting the processivity of the RT enzyme (Figure 8A; compare cDNA lengths in the standard reaction at 17 nM dTTP with those of the 26 and 34 nM reactions). Although it is clear that the 3TC-resistant RT variants are less processive than the wild-type enzyme at all dTTP levels tested, the difference is more pronounced at low dTTP concentrations. For instance, the relative activity of the 184Val variant compared with wild-type is 67% at 442 nM dTTP, but this value drops to merely 25% at 17 nM dTTP (Figure 8B). Similarly, activity of the Ile variant decreased from 51 to 17% compared with the wild-type activity. These combined data indicate that RT processivity defects are more pronounced at sub-optimal Mg^{2+} and/or dNTP levels.

Reduced fitness of the 3TC-resistant HIV-1 variants in primary cells

The intracellular dNTP level varies significantly between different cell types and fluctuates during the cell cycle (Bray and Brent, 1972; Cohen *et al.* 1983; Dahbo and Eriksson, 1985; Meyerhans *et al.*, 1994). Interestingly, the intracellular dNTP concentration was reported to be particularly high in established T cell lines that we used in the initial replication experiments (Figure 1). Based on the dNTP sensitivity of the RT processivity defect (Figure 8), replication studies should ideally be performed in cell types with a low dNTP pool. We therefore set out to test the replication potential of 184 variant viruses in primary lymphoid cells. Before evaluating the effect of dNTP pool level on replication of the 3TC-resistant HIV-1 viruses, we analysed the intracellular dNTP concentration in both

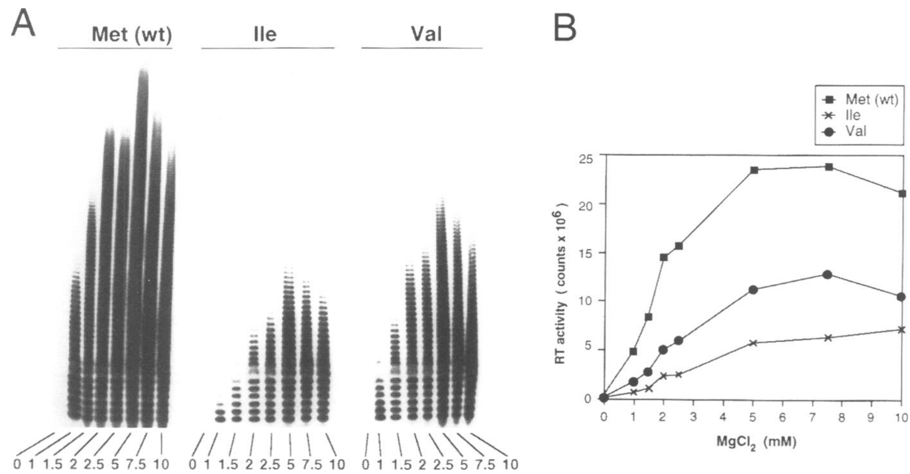

Fig. 7. Effect of $MgCl_2$ concentration on processivity of the 3TC-resistant RT variants. RT assays were performed with the poly(rA)·oligo(dT) template–primer and increasing amounts of $MgCl_2$ (0, 1, 1.5, 2, 2.5, 5, 7.5 and 10 mM), indicated below (A). cDNA products were fractionated on a gel and visualized by autoradiography (**A**). Reaction samples were also spotted onto DE81 paper and quantitated as described in Materials and methods (**B**).

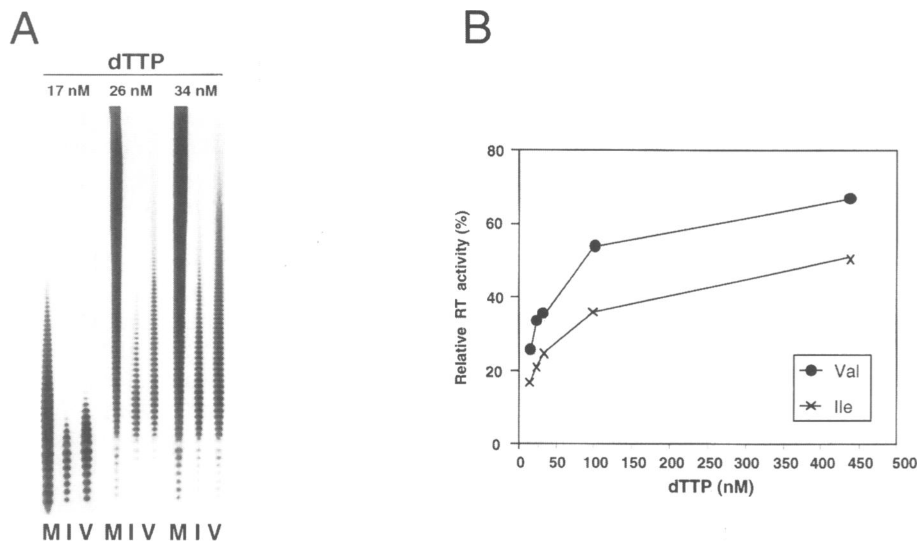

Fig. 8. Effect of dNTP concentration on the processivity of 3TC-resistant RT enzymes. The three virion-derived RT enzymes were assayed on the poly(rA)·oligo(dT) template–primer with varying dTTP levels (17, 19, 26, 34, 102, 442 nM). Reaction products were analysed by electrophoresis on a denaturing polyacrylamide gel (**A**); shown are the 17, 26 and 34 nM dTTP incubations and cDNA synthesis was quantitated on DE81 filters as described in Materials and methods. (**B**) The relative activity of the 184Val and Ile mutants compared with the wild-type 184Met enzyme, with the latter activity set at 100% for each dTTP concentration tested.

the T cell line SupT1 and peripheral blood mononuclear cells (PBMCs). The data are summarized in Table II. Consistent with previous reports, dNTP levels were ~20-fold higher in the established T cell line compared with the primary cells. The characteristic asymmetry of dNTP pools, with dCTP and dGTP being the lowest, was observed in both cell cultures. We also measured dNTP levels in phytohaemagglutinin (PHA)-stimulated PBMC that were used in HIV-1 infection experiments. PHA activation only marginally increased the intracellular dNTP pool (Table II).

Activated PBMCs were transfected with the set of 184-mutated HIV-1 plasmids and CA-p24 production was measured for several weeks (Figure 9). Replication of the two 3TC-resistant variants was delayed compared with the wild-type virus. Furthermore, a significant decrease in the amount of virus produced at the peak of infection was apparent for the Ile variant, and to a lesser extent for the Val variant. To obtain further evidence for a replication defect of the Val variant, we performed sensitive competition experiments with a mixture of this variant and the wild-type virus. Whereas the wild-type outcompeted the

Table II. Intracellular dNTP pools

Cell type	dNTP concentration (nmol/10^9 cells)[a]			
	dGTP	dATP	dTTP	dCTP
SupT1 T cell line	19	50	58	14
PBMC primary cells	n.d.[b]	1.5	1.8	0.7
PBMC, PHA-activated cells	n.d.[b]	2.5	4.9	0.6

[a]Values represent the average of two independent measurements.
[b]n.d. = non-detectable.

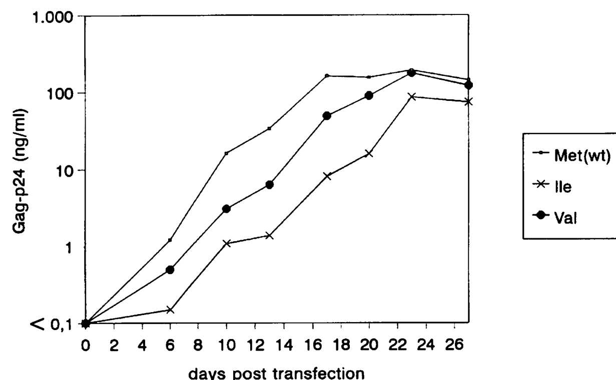

Fig. 9. Impaired replication of 3TC-resistant HIV-1 variants in primary PBMC cells. PHA-stimulated peripheral blood mononuclear cells were transfected with 10 μg of the 184 variant molecular clones. CA-p24 antigen levels were measured in the culture supernatant twice a week and followed for 4 weeks.

3TC-resistant variant within two passages on PBMCs (± 35 days), a mixture remained for at least 10 passages (≥7 weeks) in SupT1 cultures. These results, combined with the SupT1 replication experiments presented in Figure 1, indicate that replication of the 3TC-resistant HIV-1 variants is specifically restricted in the primary cell type.

We propose that the low dNTP pool causes this cell type-specific defect of 3TC-resistant virus in primary cells. However, we cannot formally exclude that the variant RT enzymes present in PBMC-produced virions are more defective than the corresponding RT samples from SupT1-derived virions. To test this, we extracted the RT protein from PBMC virions and performed poly(rA)·oligo(dT) assays. We measured the characteristic (Met>Val>Ile) activity/processivity pattern with the PMBC samples (data not shown), indicating that the cell type differences are not manifest at the level of the RT protein.

Discussion

Individuals treated with 3TC develop resistance within 2 weeks, at which time point a stable Met184Val substitution (ATG→GTG) can be detected (Schuurman *et al.*, 1995; Wainberg *et al.*, 1995). However, patient samples taken at earlier times (± 1 week) frequently contained another mutation at the same codon position (Met184Ile, ATG→ATA) (Schuurman *et al.*, 1995). Our results indicate that processivity defects measured *in vitro* for 3TC-resistant RT enzymes correlate with a reduced replication rate of the corresponding HIV-1 variants in primary cell types. The ranking order of fitness (Met>Val>Ile) explains the outgrowth of the 184Val virus in 3TC-treated patients. Both RT variants have been demonstrated to provide the drug resistance phenotype (Boucher *et al.*, 1993; Tisdale *et al.*, 1993). The initial appearance of the 184Ile variant is likely to be caused by the high frequency of G→A transitions at this codon position (Keulen *et al.*, 1996, and our unpublished results). Eventual outgrowth of the 184Val virus is consistent with the better enzyme property and superior replication capacity of this variant.

In contrast to the studies in primary cells, no replication defect was apparent in tissue culture infections with the T cell line SupT1. Recently, similar findings were reported with the 184 variants cultured on the MT-4 T cell line (Larder *et al.*, 1995). Based on the observation that the processivity defect of 184-mutated RT is more pronounced at low dNTP levels (Figure 8), we propose that the discrepancy between virus replication capacity of the 3TC-resistant viruses in T cell lines versus primary cells is caused by the low dNTP concentration in the latter cell type (Table II). Other findings support the idea that HIV-1 replication is restricted in cell types with a low dNTP pool. For instance, primary T lymphocytes were reported to restrict replication of a wild-type HIV-1 isolate at the level of reverse transcription, most probably due to limiting dNTPs (Zack *et al.*, 1990, 1992). Virus production was restored upon stimulation of these cells with mitogen (Zack *et al.*, 1990, 1992), a treatment that stimulates the intracellular dNTP pools (Meyerhans *et al.*, 1994). Furthermore, HIV-1 replication can be inhibited by drugs that reduce the level of at least one of the dNTP building blocks (Gao *et al.*, 1993b; Lori *et al.*, 1994; Meyerhans *et al.*, 1994). Thus, we recommend that primary cell types should be used in fitness assays with drug-resistant variants of the HIV-1 virus.

We cannot currently rule out that other factors influence the process of reverse transcription in a cell type-specific manner. Such accessory factors may be packaged into the virion to exert an effect on the reverse transcription mechanism. For instance, the architecture of retroviral particles, which is designed to optimize reverse transcription, may be different in composition and/or structure depending on the host cell type. Co-factors that keep the polymerase clamped to the template have been reported for the T7 DNA polymerase (Kornberg and Baker, 1992; Kunkel *et al.*, 1994) and the RNA polymerases of polio virus (Paul *et al.*, 1994) and influenza virus (Shimizu *et al.*, 1994). However, we and others measured activity and processivity defects with purified, recombinant forms of the mutant RT protein (Wakefield *et al.*, 1992; Boyer and Hughes, 1995) (data not shown), indicating that cellular and/or viral co-factors are not responsible for the reduced processivity of 184Val and 184Ile enzymes.

The YMDD motif is a conserved amino acid sequence in all HIV/SIV RT sequences (Myers *et al.*, 1993), although one natural HIV-1 isolate with an 184Ile residue was reported (Myers *et al.*, 1993; Gürtler *et al.*, 1994). This motif is part of the β9–β10 turn of the four-stranded antiparallel β-sheet in the polymerase active site or 'palm domain' of the RT protein and mutations in this motif have been shown to inhibit RT activity dramatically (Lowe *et al.*, 1991; Boyer *et al.*, 1992; Wakefield *et al.*, 1992). Crystal structures of RT are available, but the position of the two catalytically required Mg^{2+} ions has not been solved. The carboxylic side chains of the two Asp residues of the YMDD motif were proposed to coordinate the two metal ions, which in turn coordinate the β- and γ-phosphates of the deoxynucleoside triphosphate (dNTP) (Kohlstaedt *et al.*, 1992; Jacobo-Molina *et al.*, 1993; Pelletier *et al.*, 1994; Sawaya *et al.*, 1994). Furthermore, the Cβ atom of Met184 interacts directly with the amide group of Asp185 (Rodgers *et al.*, 1995). Thus, mutation of residue 184 could affect the interaction between RT and the incoming dNTP in such a way that the 3TC drug is specifically rejected. The side chain of residue 184 may directly sense the unnatural (–) sugar group of the 3TC, but it is also possible that 3TC resistance is an indirect

effect caused by a conformational change in the catalytic domain.

All three amino acids observed at position 184 in patient isolates (Met, Val, Ile) facilitate the β-sheet conformation (Chou and Fasman, 1978). In fact, these residues are the top three β-sheet formers (Pβ = 1.67, 1.65 and 1.60, respectively), with the next residue at a large distance (Cys, Pβ = 1.30). Although residue 184 itself is part of the turn connecting β-sheets 9 and 10, these results may suggest that residue 184 is critical for initiation of the β9-sheet conformation. The importance of residue 184 is supported further by the replication defect of other 184 RT mutants (Wakefield *et al.*, 1992) and the conserved nature of this catalytic domain in all retroviral polymerases (Kamer and Argos, 1984; Argos, 1988; Pelletier *et al.*, 1994; Sawaya *et al.*, 1994). Thus, only a subtle distortion of the β-sheet is allowed, and reduced activity/processivity of the RT variants (Met>Val>Ile) may correlate with decreased stability of the β9-sheet conformation (Met> Val>Ile). A recent study by Wainberg *et al.* (1996) revealed another intriguing characteristic of the 3TC-resistant Val184 enzyme. These authors demonstrated an increase in fidelity of nucleoside insertion by this RT variant compared with wild-type enzyme. We obtained similar results with both the virion-extracted and *E.coli* RT proteins used in this study. Interestingly, a more dramatic increase in fidelity was measured for the Ile enzyme compared with the Val variant (N.K.T.Back *et al.*, our unpublished data), which correlates with the destabilizing effect on the β9-sheet.

In conclusion, we demonstrate a functional defect of 3TC-resistant RT enzymes that leads to a loss of viral fitness for replication in primary cells. *In vivo*, even a partially reduced level of virus replication will diminish the viral load, and this may be of considerable clinical benefit if the asymptomatic period can be extended and development of AIDS postponed (Coffin, 1995). There may be other drugs or drug combinations that further reduce the viral fitness. In order to assess this effect accurately in patients, long-lasting multi-centre clinical trial are required (see, for example, the Concorde trial for AZT, 1994). Finally, we would like to propose that therapy with 3TC and hydroxyurea (HU) may be a particularly potent drug combination. HU inhibits the cellular enzyme ribonucleotide reductase and thereby reduces the intracellular dNTP concentration. HU strongly inhibits HIV-1 DNA synthesis and viral replication (Gao *et al.*, 1993b; Lori *et al.*, 1994; Lori and Gallo, 1995). Since these results were obtained in experiments with activated lymphocytes, it seems plausible that the inhibitory effect would be more dramatic in quiescent cells, due to a lower baseline of the dNTP pool (Lori and Gallo, 1995). We believe that the fitness of 3TC-resistant viruses may decline further at HU-induced low dNTP conditions.

Materials and methods

Cell lines

The SupT1 T cell line was grown in RPMI 1640 medium (Gibco BRL, Life Technologies Inc.) supplemented with 10% fetal calf serum (FCS, Gibco) and antibiotics. The cells were passaged twice a week and kept in culture for a maximum of 20 passages. Donor PBMCs were prepared by Ficoll-Paque (Pharmacia Biotech) density gradient centrifugation of heparinized blood from three HIV-1 seronegative individuals. The mix was stimulated for 2–3 days with PHA (1 μg/ml) in RPMI 1640 supplemented with 10% FCS, antibiotics and polybrene (5 μg/ml) (Sigma). After transfection, the PBMC culture medium was supplemented with 10% Lymphocult IL-2 (Biotest, Soest, The Netherlands). Half of the culture suspension was replaced with fresh medium containing 2×10^6 PHA-stimulated donor PBMCs at days 7, 14 and 21 post-infection.

Plasmid manipulations and viruses

Mutant RT genes encoding the 184Ile (ATA) or 184Val substitution (GTG) were generated on an HXB2 RT fragment by site-directed mutagenesis (Kunkel, 1985; Boucher *et al.*, 1993). The 184-mutated RT fragments were cloned back into a modified HXB2 molecular clone (W.Keulen *et al.*, in preparation). Plasmid DNA was purified on a caesium chloride gradient and 10 μg was electroporated into 5×10^6 PHA-stimulated PBMCs and supplemented with 0.5×10^6 PHA-stimulated PBMCs. Alternatively, recombinant virus was generated by the co-transfection method (Kellam and Larder, 1994). In brief, 5×10^6 SupT1 cells were co-electroporated with one of the three RT fragments and the RT-deleted plasmid pHIVΔRTBstEII (Kellam and Larder, 1994). After transfection, 0.5×10^6 SupT1 cells were added and the cells were maintained as described by de Jong *et al.* (1992). Viral stocks were harvested when full-blown syncytia appeared. Cells were removed by centrifugation at 1000 *g* for 10 min and the supernatant was filtered through a Millipore filter (0.45 μM). Infectious titres were determined by endpoint titration on the C8166 cell line as described (Schmidt, 1979). Infections were performed with an m.o.i. of 10^{-4}, 10^{-3} and 10^{-2}. Supernatant was harvested daily and measured for CA-p24 antigen production and RT activity. The infectious titre was determined by plaque-forming units on HeLa-CD4+ cells (HT4lacZ-1) (Rocancourt *et al.*, 1990; Larder *et al.*, 1990). The 184 mutations were also introduced into an AZT-resistant RT gene derived from a patient (containing the M41L and T215Y amino acid changes). The RT fragments subsequently were co-transfected with the RT-deleted HXB2 plasmid to produce recombinant viruses. Replication experiments were assayed by CA-p24 antigen production and RT activity.

CA-p24 assay

Culture supernatants were heat inactivated for 30 min at 56°C in the presence of Empigen-BB (final concentration of 0.05%) (Calbiochem, La Jolla). CA-p24 concentration was determined by twin-site enzyme-linked immunosorbent assay (ELISA) with D7320 (Biochrom, Berlin) as the capture antibody, alkaline phosphatase-conjugated α-p24 MAb (EH12-AP) and the AMPAK amplification system (Dako Diagnostics LTD, ITK diagnostics BV) as previously described (Moore *et al.*, 1990; McKeating *et al.*, 1991). Recombinant p24, expressed in a baculo vector, was used as a reference standard.

Reverse transcriptase assays

Wild-type and variant RT supernatants were stored at –70°C and the enzymes were released from the virions by NP-40 treatment (0.5% final concentration) for measuring the RT activity in a poly(rA)·oligo(dT) assay (Willey *et al.*, 1988). Reaction mixtures contained 60 mM Tris (pH 7.8), 75 mM KCl, 5 mM $MgCl_2$, 0.1% NP-40, 1 mM EDTA, 5 μg/ml poly$(rA)_{7000}$, 0.16 μg/ml oligo$(dT)_{15}$, 4 mM dithiothreitol (DTT) and 50 μCi/ml [^{32}P]dTTP (3000 Ci/mmol). Samples were incubated at 37°C and 10 μl aliquots were taken after 3 h and spotted onto DEAE ion-exchange paper (DE81 paper, Whatman). The filter was washed three times in 5% Na_2HPO_4 in order to remove unincorporated [^{32}P]dTTP, and dried after two 96% ethanol washes. The spots were visualized by autoradiography and quantitated on a PhosphorImager. The length distribution of the cDNAs synthesized with a poly(rA)·oligo(dT) template–primer complex was analysed on a 6.0% polyacrylamide–7.1 M urea sequencing gel.

The poly(rC)·oligo(dG) trap (Amersham) was added to a pre-formed RT–poly(rA)·oligo(dT) complex before addition of [^{32}P]dTTP. The divalent cation Mg^{2+} in the standard RT assay was replaced by Mn^{2+} in the form of 0.5 mM $MnCl_2$ (Sigma). Stability of the RT enzyme was assayed in extraction buffer (RPMI 1640 culture medium + 0.5% NP-40) for either a variable time at different temperatures.

As heteropolymeric template, we used M13mp18 single-stranded DNA with an HIV-1 *tat* gene insert in combination with the 18 nucleotides long –21 sequencing primer.

Western blot analysis

Western blotting was performed on pelleted virus particles. In brief, viruses purified from 10 ml of culture supernatant were solubilized in 500 μl of RPMI medium. A 30 μl sample was mixed with 6 μl (5×

concentrated) of SDS sample buffer, boiled, separated on a 10% SDS–polyacrylamide gel, and electrophoretically transferred to nitrocellulose. The immunoblot was stained either with a pool of human HIV immunoglobulins (HIVIG) or a 1:1 mixture of the RT monoclonal antibodies 3019 and 716. These reagents were kindly provided by Drs A.Prince, D.Hellund, A.M.Szilvay and P.Yoshihara through the NIH and MRC AIDS Repositories.

dNTP measurements

Two days prior to dNTP extraction, SupT1 cells were split 1 in 10. PBMCs were cultured either in the absence or presence of PHA as activator. Cell numbers were determined with a Coulter Counter. SupT1 cells and PBMCs (50×10^6 cells each) were collected by low-speed centrifugation and (d)NTPs were extracted with 0.4 M $HClO_4$ on ice. After 10 min, the samples were centrifuged (11 000 *g* at 4°C for 5 min) and the resulting supernatants were removed and neutralized to pH 7 by addition of 5 M H_2CO_3. Deoxynucleotides were detected by using a periodate-oxidation procedure (Garrett and Santi, 1979) and analysed by anion-exchange HPLC (de Korte *et al.*, 1985) with the following modifications: deoxynucleotides were separated on a Partisphere Sax column (125×4.6 mm, 5 μM particle size, Whatman International Ltd) with a gradient of 7.5–750 mM NaH_2PO_4, pH 4.55 at flow rate of 1.0 ml/min. UV detection was at 230, 254, 262 and 280 nm with a Gynkotek UVD 340 detector. Protein concentrations were determined as described (Smith *et al.*, 1985) with human serum albumin as a standard.

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