JOURNAL OF VIROLOGY, July 2001, p. 5772–5777 Vol. 75, No. 13
0022-538X/01/$04.00+0 DOI: 10.1128/JVI.75.13.5772–5777.2001

Exploitation of the Low Fidelity of Human Immunodeficiency Virus Type 1 (HIV-1) Reverse Transcriptase and the Nucleotide Composition Bias in the HIV-1 Genome To Alter the Drug Resistance Development of HIV

JAN BALZARINI,[1]* MARIA-JOSÉ CAMARASA,[2] MARIA-JESUS PÉREZ-PÉREZ,[2] ANA SAN-FÉLIX,[2] SONSOLES VELÁZQUEZ,[2] CARLO-FEDERICO PERNO,[3] ERIK DE CLERCQ,[1] JOHN N. ANDERSON,[4] AND ANNA KARLSSON[5]

Rega Institute for Medical Research, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium[1]; Instituto de Química Médica, Consejo Superior Investigaciones Cientificas (CSIC), 28006 Madrid, Spain[2]; Department of Experimental Medicine, University of Rome "Tor Vergata," I-00135 Rome, Italy[3]; Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907[4]; and Karolinska Institute, Division of Clinical Virology F68, Huddinge University Hospital, S-141 86 Huddinge/Stockholm, Sweden[5]

Received 27 December 2000/Accepted 30 March 2001

The RNA genome of the lentivirus human immunodeficiency virus type 1 (HIV-1) is significantly richer in adenine nucleotides than the statistically equal distribution of the four different nucleotides that is expected. This compositional bias may be due to the guanine-to-adenine (G→A) nucleotide hypermutation of the HIV genome, which has been explained by dCTP pool imbalances during reverse transcription. The adenine nucleotide bias together with the poor fidelity of HIV-1 reverse transcriptase markedly enhances the genetic variation of HIV and may be responsible for the rapid emergence of drug-resistant HIV-1 strains. We have now attempted to counteract the normal mutational pattern of HIV-1 in response to anti-HIV-1 drugs by altering the endogenous deoxynucleoside triphosphate pool ratios with antimetabolites in virus-infected cell cultures. We showed that administration of these antimetabolic compounds resulted in an altered drug resistance pattern due to the reversal of the predominant mutational flow of HIV (G→A) to an adenine-to-guanine (A→G) nucleotide pattern in the intact HIV-1-infected lymphocyte cultures. Forcing the virus to change its inherent nucleotide bias may lead to better control of viral drug resistance development.

The genomes of retroviruses display striking differences in nucleotide composition, which is an important factor in determining the unusual compositions of retroviral proteins (6, 8, 22, 23). For example, the genomes of lentiviruses such as human immunodeficiency virus (HIV) are highly rich in A, less rich in G, and markedly deficient in C. Thus, proteins of HIV are rich in lysine and other polar amino acids encoded by A-rich codons and low in proline, which is encoded by C-rich codons. The extreme compositional differences extend into all major proteins of the viruses, from the hypervariable polypeptides that comprise the viral envelope to the conserved domain of reverse transcriptase (RT). The magnitude and dispersion of these effects make it likely that the variation in protein composition driven by the biased nucleotide frequencies is an important factor in shaping the characteristic phenotypes of the different viral lineages.

HIV type 1 (HIV-1) appears to be among the most rapidly evolving genetic elements known, and the A-biased genome seems to have the potential to contribute to this process (2, 8, 9, 13). The bias may also play a role in producing the surprisingly large proportion of nucleotide substitutions that cause amino acid changes in HIV proteins (30), since it favors G-A transitions over T-C transitions, which tend to promote interchanges among polar residues encoded by A- and AG-rich codons. Such replacements are expected to have minimal deleterious effects on protein function and consequently should produce large numbers of viable variants in the population. Genes that encode antigens (virulence factors) for many bacterial, protozoan, and metazoan pathogens also display the unusual A bias, which is reflected in the composition of the encoded proteins, making it likely that diverse pathogens employ similar mechanisms for the generation of variation (13). The G→A hypermutability has been explained by the asymmetric endogenous deoxynucleotide triphosphate (dNTP) pools, with the dCTP and dGTP pools being the lowest and the dCTP/dTTP ratios being on the order of 1:2 to 1:6 (28). Thus, the G→A hypermutation found in the HIV-1 genome has been directly linked to a dCTP pool imbalance during reverse transcription (26, 34, 35).

The low fidelity of HIV RT is also responsible for the high mutation rate, and, thus for the marked extent of variation within the HIV genome, leading to the swarm of HIV-1 quasispecies that is present in each patient. The mutation rate of HIV-1 has been estimated to be approximately $\sim 3.4 \times 10^{-5}$, which is an average of ~1 mutation per replication cycle (12, 27, 33, 37). These properties of HIV are thought to be the

* Corresponding author. Mailing address: Rega Institute for Medical Research, Minderbroedersstraat 10, B-3000 Leuven, Belgium. Phone: (32) 16 337341. Fax: (32) 16 337340. E-mail: jan.balzarini@rega.kuleuven.ac.be.

cause of the relatively fast emergence of drug-resistant HIV-1 strains in cell culture and in the clinical setting.

We hypothesized that the low fidelity of HIV-1 RT on the one hand and the adenine nucleotide hypermutability bias on the other hand could be exploited to manipulate and redirect the mutational pattern of resistance of HIV-1 to antiviral drugs by influencing the dNTP pools of the target cells. To provide experimental evidence for this novel concept, we have used TSAO derivatives (i.e., TSAO-m^{3}T [10, 32] and TSAO-5-dimethylamido-1,2,3-triazole [referred to as TSAO-triazole] [1, 38]) that belong to the class of nonnucleoside RT inhibitors (NNRTIs). These drugs have the following characteristics that are ideally suited to serve our purpose. First, TSAO-m^{3}T is a highly HIV-1-specific NNRTI that is nontoxic to human cells at concentrations that are 3 orders of magnitude higher than its antivirally effective concentration in cell culture (50% effective concentration [EC_{50}], ~0.05 μM) (3). Second, administration of TSAO-m^{3}T to HIV-1-infected human lymphoblast CEM cultures results in a relatively rapid emergence of drug-resistant HIV-1 strains (4). Third, and most importantly, TSAO derivatives consistently select for the Glu138Lys mutation (herein referred to as the 138Lys mutation) in HIV-1 RT that results from a transition mutation of codon GAG to codon AAG. No mutations in other codons of the RT gene of HIV-1 have ever been observed in TSAO-treated virus-infected (CEM) cell cultures (5). It should be noted that the 138Glu (GAG)→Lys (AAG) mutation adheres to the biased G→A hypermutability that has proven to be characteristic for lentiviruses such as HIV. In an attempt to counteract the hypermutability bias of G→A, we have tried to reverse the imbalance of the natural intracellular ratio of dCTP and dTTP pools by the addition of 2′-deoxycytidine (dCyd) and the dCyd deaminase inhibitor tetrahydrouridine (THU) to the selection medium. We demonstrated that under these experimental conditions, the mutational bias of G→A could be not only counteracted but even reversed, resulting in the appearance of a novel amino acid mutation at position 138 of the RT upon TSAO exposure of the HIV-1-infected cell cultures. Moreover, forcing the virus to induce a novel mutation in its RT resulted in lower levels of resistance of the mutated HIV-1 to the TSAO derivatives.

MATERIALS AND METHODS

Drugs. The following TSAO derivatives were used in our study: TSAO-T (1-[2′,5′-bis-*o*-(*t*-butyldimethylsilyl)-(β-D-ribofuranosyl)thymine]-3′-spiro-5″-[4″-amino-1″,2″-oxathiole-2″,2″-dioxide]), the N^3-methyl derivative of TSAO-T (designated TSAO-m^{3}T), and TSAO-triazole (10, 32, 38).

Selection of mutant HIV-1 strains. HIV-1(III_B) was subject to serial passages in 5-ml CEM or MT-2 cell cultures (~3×10^5 cells/ml) in the presence of the TSAO derivatives and one of the following compounds: dCyd, THU, thymidine (dThd), mycophenolic acid, 2′-deoxyformycin, and hydroxyurea. Drug concentrations were as follows: TSAO-triazole, 1 μg/ml; TSAO-m^{3}T, 0.5 μg/ml; dCyd, 0.5 mg/ml; THU, 100 μg/ml; dThd, 1 μg/ml in experiments 1 and 2 and 2 μg/ml in experiment 4; 2′-deoxycoformycin, 1 μg/ml; hydroxyurea, 1 μg/ml; and mycophenolic acid, 0.05 μg/ml. TSAO-triazole and TSAO-m^{3}T were added at the time of each subcultivation (1:10) (i.e., every third or fourth day of cultivation). The drugs (except TSAOs) were used at their optimal subtoxic concentrations. Suboptimal dCyd and THU levels were not investigated in our study. THU was added to dCyd to diminish deamination of dCyd and, thus, to keep dCyd-derived dCTP pools as high as possible throughout the experiment. Except for the TSAO derivatives the other drugs were added each day. Mutant virus breakthrough became visible as syncytium formation in the CEM or MT-2 cell cultures and was estimated as the percentage of the cell culture that contained HIV-1-induced syncytia. HIV-1-infected CEM or MT-2 cell cultures that were not exposed to the test compounds served as the control. The numbers of giant cells that appeared in these HIV-1-infected control cell cultures 3 to 4 days postsubcultivation were estimated microscopically and arbitrarily designated 100%. Generally, an average of 20 to 40 syncytia per microscopic field (magnification, ×400) was observed.

Determination of the amino acid sequence of the RT of mutant HIV-1 strains. CEM cells (3×10^5 ml) were infected with mutant HIV-1 strains at 200 50% cell culture infective doses ($CCID_{50}$) and incubated in RPMI 1640 culture medium for 3 days at 37°C. Then the cells were centrifuged and washed twice with phosphate-buffered saline in 1.5-ml Eppendorf tubes. To 10^6 CEM cells, 100 μl was added containing 10 μl of PCR buffer (concentrated 10 times; 100 mM Tris-HCl [pH 8.3], 500 mM KCl, 15 mM $MgCl_2$, and 0.01% [wt/vol] gelatin) (Cetus-Vanderheyden, Brussels, Belgium), 8 μl $MgCl_2$ (25 mM), 72 μl of Milli-Q water, and 10 μl of proteinase K (10 μg) (Calbiochem) in 0.5% Tween 20 and 0.5% NP-40 in H_2O. The cell suspension was then incubated at 56°C for 1 h and subsequently heated at 95°C for 10 min. The samples were stored at −20°C before PCR analysis. Amplification of proviral DNA (35 cycles) was performed with an extract from 10^5 cells in a solution containing 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 2.5 U of thermostable DNA polymerase (Dyna Zyme; Finnzymes, Inc.), and a 15 μM concentration of each primer in a final volume of 100 μl. The first set of primers (5′-GTAGAATTC TGTTGACTCAGATTGG and 5′-TTCTGCCAGTTCTAGCTCTGCTTCT) gave a 900-bp product of the proviral RT gene. One-tenth of the reaction product from the negative samples from the first PCR was then transferred as a template to a new 35-cycle PCR with a second set of primers (5′-CCTGAAAA TCCATACAATACTCCAGTATTTG and 5′-AGTGCTTTGGTTCCTCTAAG GA-GTTTAC), giving a 727-bp RT fragment covering amino acids 50 to 270. The second set of oligonucleotides primes internally from the first set of oligonucleotides and thereby amplifies specific products from the first PCR, whereas unspecific products are not further amplified. The PCR products were made visible on a 1% agarose gel.

Testing of sensitivity of wild-type and mutant HIV-1 strains against HIV-1-specific test compounds. CEM cells were suspended at 250,000 cells/ml in culture medium and infected with wild-type HIV-1(III_B) and mutant 138Lys and Glu138Gly (herein referred to as 138Gly) strains at 100 $CCID_{50}$/ml. Then, 100 μl of infected cell suspension was added to 200-μl microtiter plate wells containing 100 μl of an appropriate dilution of the test compounds. After 4 days of incubation at 37°C, the cell cultures were examined for syncytium formation as previously described (3).

Competition experiments between mutant 138Lys and 138Gly RT virus strains. One milliliter of a CEM cell culture (2×10^5 cells/ml) was infected with equivalent infective doses of 138Lys and 138Gly RT mutant viruses in a 48-well microtiter plate. The infective doses of the viruses used in our experiments were determined by virus titration to be the highest dilution of the virus that gave complete cytopathicity after 5 days of incubation at 37°C in a humidified CO_2-controlled incubator. This virus dose reflects a low multiplicity of infection and is estimated to be ~10 $CCID_{50}$. The competition experiments were carried out in the absence of drug to reveal the inherent fitness differences between the different mutant virus strains. Cell culture passages were performed every 3 to 4 days by adding 0.1 ml of the infected cell cultures to 0.9 ml of fresh cell culture medium containing CEM cells. At each passage, the infected cell cultures were suspended prior to their transfer (culture supernatant plus cells) to the fresh CEM cell cultures. After 4 and 11 subcultivations, 5-ml cell cultures were prepared for analysis. Three milliliters was used for DNA preparation using the QIA Amp blood kit (Qiagen, Westburg, Leusden, The Netherlands), and the remainder of the cell culture was frozen at −80°C. The DNA sequence corresponding to amino acid position 138 of the RT was determined by direct cycle sequencing on an ABI Prism 310 sequencer (Applied Biosystems, Foster City, Calif.) using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems).

RESULTS

Selection of TSAO-resistant HIV-1 strains in the presence of dCyd. Two different TSAO derivatives (TSAO-m^{3}T and TSAO-triazole) were exposed to HIV-1-infected CEM or MT-2 cell cultures in the absence or presence of dCyd plus THU. The TSAO derivatives were present at a fixed concentration (~20-fold their EC_{50}s) throughout the selection experiment. After two to four subcultivations, HIV-1-induced giant

TABLE 1. Breakthrough of virus-induced cytopathicity in drug-treated CEM or MT-2 cell cultures exposed to HIV-1 (III_B)

Expt no.	Drug(s)	Cell line	50% breakthrough of virus-induced cytopathicity on day:							
			0	3	6–7	10–11	14–15	19–20	22–23	25–26
1	TSAO-triazole alone	CEM	0	3	37	100	100	100	100	100
	TSAO-triazole with:									
	dCyd and THU	CEM	0	12	18	50	100	100	100	100
	dThd	CEM	0	1	18	75	100	100	100	100
2	TSAO-triazole alone	CEM	0	0	0	6	12	100	100	100
	TSAO-triazole with:									
	dCyd and THU	CEM	0	0	0	6	12	50	100	100
	dThd	CEM	0	0	0	6	12	100	100	100
3	TSAO-triazole alone	CEM	0	0	0	6	25	100	100	100
	TSAO-triazole with:									
	dCyd and THU	CEM	0	0	1	3	12	50	100	100
	Mycophenolic acid	CEM	0	0	1	3	25	100	100	100
4	TSAO-m^3T alone	MT-2	0	0	1	6	6	100	100	100
	TSAO-m^3T with:									
	dCyd and THU	MT-2	0	0	0	0	0	0	75	100
	dThd	MT-2	0	0	1	0	0	12	100	100
	2′-Deoxycoformycin	MT-2	0	0	0	12	50	100	100	100
	Hydroxyurea	MT-2	0	0	0	6	100	100	100	100

cells appeared in the cell cultures (Table 1). HIV-1 variants in which the Glu138 codon (GAG) was mutated to the Lys codon (AAG) emerged in the HIV-1-infected cell cultures where TSAO was present as the sole drug (Table 2). This mutation followed the hypermutational bias (G→A), since the first nucleotide of codon 138 had mutated from G to A, resulting in the Lys (AAG) codon. However, in the cell cultures where TSAO was present in combination with dCyd plus THU, a novel mutation at codon 138 of the HIV-1 RT gene consistently emerged. Instead of 138Lys (AAG), the mutant HIV-1 variants now contained 138Gly (GGG) in the HIV-1 RT. The middle nucleotide of codon 138 had now mutated from A to G, resulting in the Gly (GGG) codon. TSAO combinations with other antimetabolites that influence the purine nucleotide pool ratios (such as mycophenolic acid and 2′-deoxycoformycin, which afford higher dATP/dGTP pool ratios, or hydroxyurea, which confers higher dGTP/dATP pool ratios) or combination of TSAO with dThd (which should increase the dTTP/dCTP pool ratios) did not result in the counteracting mutational effect as shown for the combination of TSAO plus dCyd plus THU. In all these cases, the Lys (AAG) mutation appeared at codon 138 of the HIV-1 RT gene.

Mutant virus breakthrough in the presence of TSAO versus TSAO plus antimetabolites. There seemed to be a slight delay of drug-resistant virus breakthrough when TSAO was combined with dCyd and THU than when TSAO was administered as a single drug or combined with other antimetabolites that did not increase the dCTP/dTTP pool ratio (Table 1). At least one additional subcultivation (for the CEM cell cultures) or two additional subcultivations (for the MT-2 cell cultures) were required to afford fulminant (100%) cytopathicity when dCyd plus THU was added to the TSAO-treated cultures. The slight delay of TSAO resistance development in the presence of dCyd plus THU could have been a function of a lower replication rate of the virus in the presence of perturbed nucleotide pools. However, to reveal whether the slight delay of virus breakthrough might have been related to a decreased fitness of the 138Gly RT HIV-1 variant over the 138Lys RT HIV-1 variant, competition of 138Gly with wild-type and 138Lys RT mutant virus strains was carried out in CEM cell cultures. In two independent experiments the 138Gly RT HIV-1 variant became the predominant virus strain upon prolonged subcultivation of these virus-infected cell cultures in the presence of 138Lys RT HIV-1 given at equal infective doses. The predominant appearance of 138Gly RT mutant virus was already visible after four passages of the virus-infected cell cultures as judged by the height of the sequence peaks. When the 138Gly RT HIV-1 variant was exposed to CEM cell cultures in the presence of wild-type virus at equal infective doses,

TABLE 2. Nature of the codon corresponding to amino acid position 138 of HIV-1 RT for wild-type or mutant HIV-1 strains

Expt no.	Drug	Nature of HIV-1 RT codon 138		
		Wild type	TSAO drug pressure	TSAO plus dCyd and THU drug pressure
1	TSAO-triazole	GAG (Glu)	AAG (Lys)	GGG (Gly)
2	TSAO-triazole	GAG (Glu)	AAG (Lys)	GGG (Gly)
3	TSAO-triazole	GAG (Glu)	AAG (Lys)	GGG (Gly) and AAG (Lys)[a]
4	TSAO-m^3T	GAG (Glu)	AAG (Lys)	GGG (Gly)

[a] A mixture of mutant 138Gly and 138Lys RT was found in the drug-exposed HIV-1-infected cell cultures.

TABLE 3. Resistance spectra of mutant HIV-1 strains in CEM cell cultures

Compound	EC_{50} (μM) for strain:		
	HIV-1 (Glu138)	HIV-1 (138Lys)	HIV-1 (138Gly)
TSAO-m^3T	0.035	>20	1.2
TSAO-T	0.024	>3	0.76
TSAO-triazole	0.05	20	3.8
dCyd	>100	>100	>100
dCyd plus THU and dTHU	>100	>100	>100
TSAO-m^3T plus dCyd plus THU	0.030	>20	1.9
TSAO-T plus dCyd plus THU	0.016		1.3
TSAO-triazole plus dCyd plus THU	0.04		4.5
Nevirapine	0.10	0.32	0.29
Emivirine	0.002	0.060	0.033
Delavirdine	0.008	0.022	0.014
Efavirenz	0.003	0.004	0.003
ddGuo[a]	2.6	4.0	3.7

[a] ddGuo, 2′,3′-dideoxyguanosine.

the wild-type virus became the predominant virus strain upon prolonged (i.e., ~11) subcultivations (see also reference 31).

Sensitivity of mutated RT virus strains to TSAO derivatives and other NNRTIs. Three different TSAO derivatives and a variety of NNRTIs were evaluated for their inhibitory effects against wild-type and 138Lys and 138Gly mutant RT HIV-1 strains. Like the 138Lys mutant virus, the 138Gly mutant virus was found to display less sensitivity to TSAO derivatives than the wild-type virus (Table 3), but the 138Gly mutant virus showed a markedly weaker profile of resistance to TSAO derivatives than the 138Lys mutant virus. In contrast, the 138Gly mutant virus was not more resistant to other NNRTIs, such as the clinically approved nevirapine, delavirdine, or efavirenz, than the 138Lys mutant virus (Table 3). Also, the nucleoside RT inhibitor 2′, 3′-dideoxyguanosine showed a similar inhibition of virus-induced cytopathicity regardless of the nature of the amino acid at position 138 of the RT. We have also confirmed the differential inhibitory effects of TSAO derivatives versus NNRTIs and dideoxyguanosine on the mutant 138Gly and 138Lys RT viruses with recombinant HIV-1 strains in which the 138Gly and 138Lys mutations were introduced through site-directed mutagenesis and recombinant virus technology (31). To reveal whether the presence of dCyd might have a direct potentiating effect on the degree of resistance of the virus strains to the TSAO derivatives, which could have explained the emergence of mutated 138Gly RT HIV-1 strains, the effect of dCyd or dCyd plus THU on TSAO sensitivity of the wild-type and mutant HIV-1 strains was measured. No influence of dCyd or dCyd plus THU on the sensitivities of the wild-type and 138Lys and 138Gly mutant HIV-1 to different TSAO derivatives was observed (Table 3). Thus, by redirecting the mutational flow upon changing the dNTP pool balance by antimetabolites, it is possible to lower the level of HIV-1 resistance to certain drugs (i.e., TSAO derivatives).

DISCUSSION

At natural endogenous dCTP and dTTP levels the biased mutational (G→A) hypermutability flow went from GAG to AAG in the presence of TSAO, resulting in a pronounced resistance profile of the 138Lys (AAG) RT-mutated virus strain (Table 3). Thus, a thymine nucleotide was erroneously placed opposite the first guanine nucleotide of codon 138, resulting in the eventual misincorporation of an adenine nucleotide instead of the guanine nucleotide. However, in the presence of TSAO plus dCyd plus THU, the intracellular pyrimidine nucleotide dCTP levels increased, resulting in a higher dCTP/dTTP ratio. As a consequence, the codon GAG was erroneously transcribed as CCC instead of CTC. Eventually, further conversion to mutated positive- and negative-strand DNA and mRNA transcription from the mutated negative-strand DNA resulted in the establishment of the new GGG codon in the mutant HIV-1 RNA, placing a Gly at amino acid position 138. Thus, changing the intracellular pyrimidine nucleotide pools not only counteracted (prevented) the mutational bias (G→A) but even reversed it to an A→G mutational flow.

G→A hypermutation is particularly pronounced among the lentiviruses, but the relevance of this transition to lentivirus evolution remains poorly understood. G→A hypermutations can be clustered in small genomic segments or can encompass the entire genome (7, 14, 16, 19, 34, 35, 40). This process is facilitated by a low dCTP/dTTP ratio during minus-strand synthesis in vitro (26, 27), in permeabilized virions, and in cells in culture (36), illustrating the importance of dNTP pools in the control of viral mutation and, possibly, evolution. The process operating over time could be responsible for the A richness of the lentiviral genome. The silent codon sites in HIV-1 are highly A rich (51%) and more deficient in G (13%) than in C (17%), and the composition of these sites should more closely reflect the true mutational bias of the genome (13). The compositional nucleotide bias of the HIV genome favors G-A transitions over T-C transitions (8), and the results presented here suggest that the normal in vivo direction of this transition is from G to A since it was observed in the presence of a variety of agents (i.e., mycophenolic acid, 2′-deoxycoformycin, and hydroxyurea) that do not influence the intracellular pool of dCTP. These observations imply that intracellular levels of dCTP are normally limiting for HIV replication in the cell, and the report that dCyd stimulates HIV replication supports this view (28). It follows that the A→G transition should be favored by a high dCTP/dTTP ratio, and the results presented here support this view. An important consequence is that pref-

erential A-to-G transitions occurring over a prolonged period might then be expected to cause a loss of the A bias of the lentiviral genome and reduced virulence since the bias is considered critical for the HIV phenotype (8). This consideration raises the possibility that increasing the dCTP/dTTP ratio may be of long-term therapeutic benefit, which represents an interesting issue for further exploration.

The observation that changing the intracellular dNTP pool ratios counteracted the mutational bias (G→A) of HIV-1 RT has a number of important fundamental and clinical implications. It is generally accepted that a large variety of HIV-1 variants exists in the quasispecies swarm and that upon drug exposure a rare preexisting mutant virus strain may become the predominant virus variant if its genetic alterations confer resistance to the drug. From our experiments, however, the appearance of the 138Gly RT mutant virus cannot simply be explained by this phenomenon since dCyd itself, or TSAO plus dCyd and THU, was not more suppressive to 138Lys RT HIV-1 than to 138Gly RT HIV-1 (Table 3). It was also ascertained that the addition of dCyd plus THU did not result in a lower cellular uptake of TSAO, which could have selected for a virus strain with a lower TSAO resistance level. Indeed, we verified that 500 μg of dCyd/ml plus 100 μg of THU/ml did not lower [^{3}H]TSAO-m^{3}T uptake (0.2 μCi of TSAO-m^{3}T/ml, i.e., 3 nM) in exponentially growing CEM cell cultures at 6 or 24 h after drug exposure (data not shown). Also, it has been previously shown that lower TSAO drug levels also consistently selected for 138Lys RT mutant HIV-1 strains, and they never selected for 138Gly RT mutant HIV-1 strains when the TSAO derivatives were added as single drugs (5). Thus, our data strongly suggest that the 138Gly RT HIV-1 mutant was selected by the appearance of a new mutation that could only emerge under increased intracellular dCTP/dTTP pool ratios, and they thus demonstrate that the appearance of mutant virus strains can also arise from novel induced mutations and not necessarily result from the selection (outgrowth) of preexisting mutant virus strains. It should be kept in mind that these observations we are made in cell cultures with a viral clone [HIV-1(III_B)]. It is currently unclear whether these observations can be extrapolated to the in vivo situation.

Our observations may also have other important clinical implications. At least two different antimetabolic drugs (either alone or in combination with other drugs) have been recently introduced in clinical trials as potential anti-HIV drugs in HIV-1-infected individuals (17, 21, 24, 25). Hydroxyurea, a ribonucleotide reductase inhibitor, leads to a preferential lowering of the dATP pool levels and results in an imbalance between dATP and dGTP in favor of dGTP (28), while mycophenolic acid may result in a lowering of the dGTP pool levels and create an imbalance between dATP and dGTP in favor of dATP (21). Thus, antimetabolite drugs, by creating dNTP pool imbalances (11), may be useful to selectively alter the mutational bias in HIV-1-infected cell cultures. A long clinical experience in the treatment of other diseases exists for hydroxyurea and mycophenolic acid. In these trials, it has been shown that they can be administered to patients over a long-term period, and these drugs are now under further clinical investigation (11, 15, 18, 28). In light of our observations it would be of particular interest to carefully choose existing drugs used for HIV-1 treatment to be combined with antimetabolic drugs, such as hydroxyurea or mycophenolic acid, and investigate the potential changes in the resistance pattern that appear under such drug combination therapy. Altered mutational patterns can indeed result in more attenuated (replication-compromised or less virulent) virus strains, as observed for the mutant (Met184Val) RT virus strains that emerge under lamivudine treatment of HIV-infected patients (39). Also, an altered mutational pattern can result in a lesser degree of virus resistance and thus better suppression of the virus by the particular drugs. However, it should be noted that in this study the mutant 138Gly RT virus strain still conferred a 30-fold reduction in drug susceptibility, which is more than sufficient for the virus to easily replicate under our experimental conditions (Table 3). The appearance of unfavorable mutations that affect the efficacy of many NNRTI drugs in patients (e.g., the Lys103Asn and Tyr181Cys mutations, in which A-to-G transition mutations are involved) may become better suppressed upon coadministration of the appropriate antimetabolites (e.g., antimetabolites that increase the dTTP/dCTP pool ratio in these particular cases), and this should not be ignored. It should also be pointed out that virus resistance to antimetabolites may not easily occur, and cellular resistance, if ever emerging, should take much longer time to occur than resistance to the virus-specific drugs.

Another interesting feature is the fact that monocytes macrophages are an important reservoir of HIV, and they have the characteristic properties of resting cells (29). Monocytes macrophages have relatively low dNTP pool levels, and their dNTP pool ratios can be much more easily influenced by antimetabolites than those of replicating lymphocytes. It should be mentioned that the dCTP/dTTP pool ratios in monocytes/macrophages were determined to be ~0.40 (compared with 0.25 for CEM cells), whereas the dGTP/dATP pool ratios in monocytes/macrophages were determined to be ~1.0 (compared with 0.5 for CEM cells). Thus, it may also be important that this continuous HIV-1 source in the human body can be manipulated by antimetabolite drugs.

It is also noteworthy that erratic hypermutability has been observed not only with other lentiviruses (i.e., caprine arthritis-encephalitis virus) (40) but also with nonlentiviruses, such as hepatitis B virus (20), bacteria, and protozoa (13). Therefore, our observations may be seen in a much broader context than HIV and may be applied to several other human pathogens.

In conclusion, we have shown that it is possible to counteract the mutational bias and particularly the adenine-over-guanine nucleotide preference of HIV-1 by changing the endogenous dNTP pool levels in HIV-1-infected cells by using antimetabolic drugs. This represents an entirely novel approach to interfering with the development of resistance to anti-HIV drugs, including both nucleoside RT inhibitors and NNRTIs. The fact that antimetabolites can be used for this purpose calls for a careful implementation of these antimetabolites in well-designed combination trials with established anti-HIV drugs.

ACKNOWLEDGMENTS

We thank Ann Absillis, Lizette van Berckelaer, and Ria Van Berwaer for excellent technical help and Christiane Callebaut for dedicated editorial help.

This research was supported by the Fonds voor Wetenschappelijk Onderzoek—Vlaanderen (FWO) (Krediet no. G.0104.98), the Geconcerteerde Onderzoeksacties (GOA) (Krediet no. 00/12), the Spanish

CICYT (project no. SAF 2000-0153-C02-01), and the European Commission (projects no. BMH4-CT97-2161 and QLK2-1999-00291).

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