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HIV and human endogenous retroviruses: An hypothesis with therapeutic implications*

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The enzyme dUTP pyrophosphatase (dUTPase, EC 3.6.1.23) is essential for cellular DNA replication and cell viability by virtue of its role in reducing the availability of dUTP as a substrate for DNA polymerases. Several members of the onco- and lentivirus families of retroviruses encode dUTPases and mutant strains of these viruses defective in this enzyme exhibit suboptimal replication kinetics. Among the lentiviruses there exists a surprising phylogenetic discontinuity in the distribution of dUTPase genes: *non-primate* viruses (EIAV, CAEV, FIV, visna) contain such genes whereas the *primate* viruses (HIVs, SIVs) do not. The reason for this difference is unknown. We suggest the following explanation: (1) the nuclear and mitochondrial compartmentalization of the mammalian dUTPase, combined with the cytoplasmic location of ribonucleotide reductase, leads to the net synthesis of dUTP, together with dCTP, dGTP and dATP in the cytoplasm; (2) this combination of dNTPs serves as a "toxic cocktail" for viral replication by virtue of its ability to promote the synthesis of uracil-substituted DNA; (3) many viruses have adapted to this challenge by encoding dUTPases that are free of normal cellular regulatory constraints; and (4) the fortuitous expression of a dUTPase encoded by one or more human endogenous retroviruses (HERVs) has led to the evolutionary loss of the putative ancestral dUTPase gene of *primate* lentiviruses. Thus, we propose that efficient replication of HIV in humans depends upon expression of a dUTPase encoded by an endogenous retrovirus. If this proposal is correct, then the entry of HIV into target cells is necessary, but not sufficient, for replication of the virus in humans.

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Abbreviations: CAEV, caprine arthritis-encephalitis virus; G3PD, glyceraldehyde-3-phosphate dehydrogenase; HERV, human endogenous retrovirus; HIV, human immunodeficiency virus; HSV, herpes simplex virus; HTLV, human T-cell lymphotrophic virus; HU, hydroxyurea; FIV, feline immunodeficiency virus; EIAV, equine infectious anemia virus; MoMLV, Moloney mouse leukemia virus; MMTV, mouse mammary tumor virus; NDP kinase, nucleoside diphosphate kinase; dNTPs, deoxynucleoside-5'-triphosphates; PCR, polymerase chain reaction; RRase, ribonucleotide reductase; SIV, simian endogenous retrovirus.

Current approaches to developing anti-HIV therapeutics have focused largely on virus-encoded proteins (e.g. reverse transcriptase, protease) or cell membrane receptors to which the virus particles initially bind. Although compounds that inhibit HIV enzymes have been developed, their therapeutic effectiveness is limited by the high mutation rates of the virus which leads rapidly to the appearance of drug-resistant variants. This has prompted some investigators to search for non-HIV encoded targets for therapeutic drug development. One approach involves inhibitors of cellular deoxynucleotide (dNTP) metabolism. For example, hydroxyurea (HU), an inhibitor of ribonucleotide reductase (and consequently dNTP synthesis), effectively reduces HIV and MoMLV replication in proliferating cells [1–4]. HIV replication is also severely inhibited by treatment of cells with excess thymidine [3] which, through the allosteric properties of ribonucleotide reductase, suppresses the synthesis of dCTP. Like all retroviruses, HIV is absolutely dependent upon the host cell for the synthesis of dNTPs which are required for reverse transcription. Thus, significant disturbances in cellular dNTP metabolism caused by targeting cellular enzymes will impair HIV replication. A major advantage of this approach for HIV therapy is that it ameliorates the problem of drug resistance caused by high mutation rates. One drawback, however, is that inhibitors of vital cellular enzymes may have undesirable side effects. In this paper, we explore a related but different possibility — that a dNTP metabolic enzyme encoded by an endogenous retrovirus common among old world primate species may be a potential target for HIV therapy.

dUTPase AND RETROVIRUS REPLICATION

dUTP pyrophosphatase (dUTPase; EC 3.6.1.23) catalyses the hydrolysis of dUTP to dUMP and PP_i (Fig. 1). The biochemical function of this enzyme is twofold; first, it provides dUMP as a precursor for dTTP synthesis; secondly, it eliminates dUTP as a substrate for DNA polymerases. dUTPase is essential for cell viability in *Escherichia coli* [5] and also in the budding yeast *Saccharomyces cerevisiae* [6]. In both systems, the absence of this enzyme causes elevated dUTP levels within the cell as a result of which uracil is extensively incorporated into replicating DNA. This occurs because DNA polymerases cannot exclude dUTP as a substrate. Ultimately, this situation induces attack on the uracil-substituted DNA by uracil glycosylase-mediated DNA repair which, in turn, leads to DNA fragmentation and cell death [7–8]. In mammalian cells, chemotherapeutic agents that induce elevated cellular dUTP levels through inhibition of dihydrofolate reductase or thymidylate synthase (e.g. methotrexate, other antifolates, CB3717) also promote DNA damage and cell killing [8]. Thus, it is becoming increasingly clear that, in all cellular systems, dUTP is a toxic metabolite and dUTPase a vital enzyme.

dUTPases are encoded by a diverse array of viruses including members of the poxvirus and herpesvirus families. This is not surprising in view of the fact that these viruses also encode other enzymes involved in dNTP metabolism (e.g., thymidine kinase, ribonucleotide reductase). This complement of enzymes allows these viruses to synthesize dNTPs, and hence DNA, independently of host cell replication. In the case of HSV-1, the viral dUTPase is unnecessary for replication in proliferating cells in culture [9]. However, HSV-1 mutants defective in the enzyme (*dut*$^-$ mutants) are severely impaired for replication in mice [10]. Similar results have been found for a dUTPase-deficient poxvirus in pigs [11]. These findings are consistent with the fact that the mammalian dUTPase is regulated in a cell-cycle dependent manner [12]. Thus, in proliferating cells, the host dUTPase can compensate for the lack of a virus-encoded enzyme. In terminally differentiated, non-proliferating neural cells, which are the natural hosts for HSV-1, the cellular enzyme is not expressed. Therefore, in whole animals, the virus-encoded dUTPase becomes critical to prevent uracil incorporation during viral DNA synthesis.

Recently, it has been shown that dUTPases are also encoded by several B- and D-type oncoretroviruses and by non-primate lentiviruses [13–15]. In the case of oncoretroviruses, the dUTPase coding region lies within the *gag* gene and is synthesized as a fusion protein with the viral protease. Among lentiviruses, it lies within the *pol* gene between the RNAse H and integrase domains. In both cases, the dUTPase is synthesized as part of a larger precursor poly-

protein and is released from this protein by the action of the *gag* protease. The discovery that these retroviruses encode dUTPases is remarkable for two reasons: first, retroviral genomes are typically small and do not encode any other enzymes involved in dNTP metabolism. Second, dUTPase is an intermediate enzyme in the *de novo* dNTP metabolic pathway. Thus, it is not immediately apparent why this one enzyme should be encoded by some retroviruses. Mutant strains of equine infectious anemia virus (EIAV), feline immunodeficiency virus (FIV) and caprine arthritis-encephalitis virus (CAEV) defective in dUTPase have been constructed [16–20]. Like HSV-1 *dut*$^-$ mutants, these strains can be propagated in proliferating cultured cells, but are severely impaired for replication and cytopathicity in macrophages, the natural hosts for these viruses. Again, this may reflect the fact that the cellular dUTPase is not expressed at a high level in these non-proliferating cells and therefore, efficient virus replication is dependent upon the virus-encoded enzyme.

Given the critical role of dUTPase for lentivirus replication in their natural hosts, an important question is why do the primate lentiviruses *not* encode a dUTPase enzyme? The answer to this question is potentially important as it may uncover new aspects of HIV replication. To address this problem, it is important to consider the following questions: *what is the role of the viral dUTPases at the molecular level?; how are these viruses exposed to dUTP?;* and, *what is the difference between primate and non-primate systems which can account for the lack of a dUTPase gene in the primate lentiviruses?* Each of these questions is considered below.

THE ROLE OF RETROVIRUS dUTPases

Retrovirus dUTPases are encapsidated within mature virus particles, indicating that the enzyme acts at the earliest stages of infection [13, 15]. Given the established role of the enzyme in cellular systems, it seems likely that the function of the retrovirus enzyme is to prevent uracil incorporation into cDNAs. Reverse transcriptases can utilize dUTP as a substrate and dUTPase-deficient strains of FIV, EIAV and CAEV synthesize uracil-substituted cDNAs in macrophages [19, 20]. The consequences of uracil incorporation into cDNAs are currently unknown but may be multifaceted. For example, Steagall *et al.* [19] found that dUTPase-deficient EIAV mutants grown in primary macrophages exhibit reduced integration (2–3 fold), and severely reduced full-length and processed provirus transcripts, relative to wild-type controls. The reasons for these effects are not known but, as suggested by the authors, it could involve damage of the proviruses by uracil glycosylase-mediated excision repair and/or the impairment of gene expression. Studies using *dut*$^-$, *ung*$^-$ (uracil glycosylase) double mutants of *E. coli* and *S. cerevisiae* indicate that the stable incorporation of uracil into DNA reduces cellular transcription in general [5, 6]. This effect may result from the inability of many transcription factors to recognize uracil-substituted DNA sequences [5, 6, 19].

It has been suggested that, for retroviruses, uracil glycosylase-mediated excision repair, converging on both cDNA strands, might lead to double-strand breakage and irreversible fragmentation of the viral genome [21, 22]. This could occur just prior to, or following, integration of the cDNA into the host cell genome. Alternatively, the introduction of single-strand gaps may induce extensive recombination leading to genome rearrangements and impairment of virus gene expression.

Recently, Lerner *et al.* [23] found that a dUTPase-deficient strain of FIV exhibits a 5-fold increase in the mutation frequency relative to wild-type virus upon infection of primary macrophages. DNA sequence analysis of mutant clones recovered by PCR from infected cells showed a preponderance of G → A transition mutations, consistent with a mispairing of uracil with guanine during cDNA synthesis. The magnitude of this effect, however, seems far too low to be responsible for the severe reduction in replication observed for dUTPase-deficient FIV in primary macrophages. This is analogous to the situation in *E. coli*, where *dut*$^-$ mutants have a mutator phenotype [24], but cell death caused by dUTPase deficiency involves uracil glycosylase-mediated excision repair and DNA fragmentation [5, 24].

It has also been suggested that retroviral dUTPases facilitate the synthesis of dTTP, particularly in non-proliferating cells where cellular dUTPase levels are expected to be low [16, 18]. Thus, it has been argued that the enzyme would

enhance cDNA synthesis through an increase in dNTP pool size. This explanation, however, is inconsistent with the fact that thymidylate synthase and other cellular dNTP metabolic enzymes are also cell-cycle regulated [25, 26] and therefore the expression of the virus-encoded dUTPase is unlikely to enhance dTTP and dNTP synthesis in non-dividing cells.

On the basis of the established role of the enzyme in cellular systems, combined with the available data for viruses, it seems most likely to us that the function of the retrovirus-encoded dUTPases is to eliminate dUTP from the cytoplasm. Therefore, we believe that it plays a protective role by preventing uracil incorporation into cDNAs and the subsequent attack on this DNA by cellular repair enzymes.

HOW ARE RETROVIRUSES EXPOSED TO dUTP?

For cDNA synthesis to occur, dNTPs must be present in the cytoplasm of the cell where retroviruses undergo reverse transcription. The very existence of retrovirus-encoded dUTPases is evidence that significant levels of dUTP must exist along with other dNTPs in the cytoplasm of cells that are the natural hosts to these viruses. dUTP is synthesized from UDP *via* the combined actions of ribonucleotide reductase and nucleoside diphosphate kinase, and it is degraded by dUTPase (Fig. 1A). Thus, dUTP can exist in the cytoplasm in significant amounts only if ribonucleotide reductase is active at a time when the cellular dUTPase is not, or if the two enzymes reside, or are active, in different locations within the cell. The mammalian ribonucleotide reductase has been reported to be a cytoplasmic enzyme [27–29]. Recently, Ladner *et al.* [30] found that two distinct isoforms of the human dUTPase exist — one nuclear, the other mitochondrial. If a similar subcellular distribution of dUTPase applies to other mammalian species, the existence of a significant cytoplasmic dUTP pool and thus, the need for dUTPase genes within some retroviruses, can be explained.

Could such a differential distribution of dNTP metabolic enzymes and the existence of a cytoplasmic dUTP pool be of functional significance? On the basis of the following argument, we suggest that this may indeed be the case. In theory, the expression of ribonucleotide reductase, in the absence of dUTPase or other dNTP metabolic enzymes, can lead to the synthesis of dUTP, dCTP, dGTP and dATP in the cytoplasm of mammalian cells (Fig. 1B). Ribonucleotide reductase catalyses the reduction of all four ribonucleoside diphosphates (UDP, CDP, GDP, ADP) to deoxyribonucleoside diphosphates (dUDP, dCDP, dGDP, dADP). Nu-

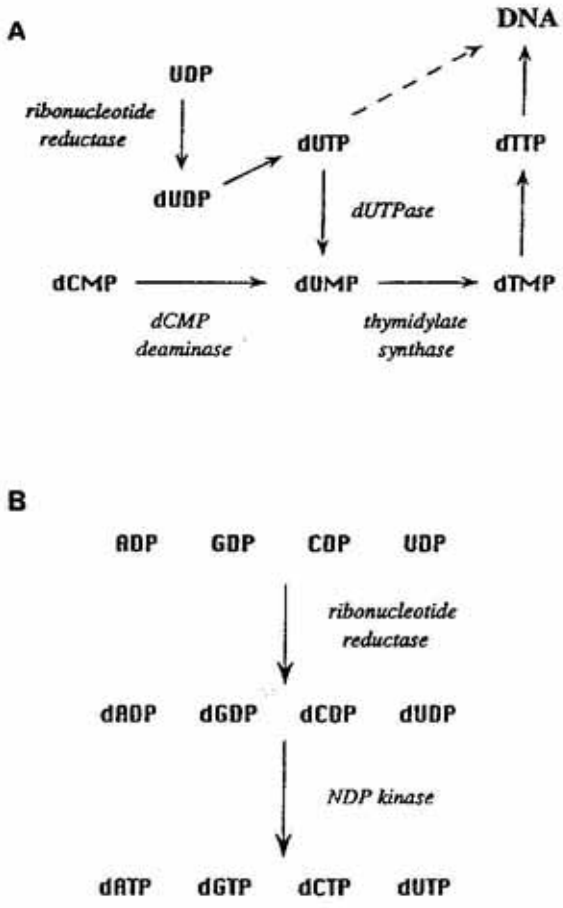

Fig. 1A. The role of dUTPase in dNTP metabolism. dUTP is formed from UDP *via* reduction of ribose by ribonucleotide reductase, and phosphorylation of dUDP by nucleoside diphosphate (NDP) kinase. dUTPase hydrolyses dUTP to yield dUMP and PP_i. In turn, dUMP, which can also be synthesized *de novo* from dCMP (*via* dCMP deaminase), is methylated by thymidylate synthase to yield dTMP. Since dUTP can be utilized as a substrate by DNA polymerases, the activity of dUTPase is critical for cell survival.

1B. The synthesis of the proposed "toxic cocktail" of cytoplasmic dNTPs. The expression of ribonucleotide reductase in the cytoplasm, along with NDP kinase, would lead to the synthesis of dUTP, along with dCTP, dATP and dGTP. In the absence of dUTPase, this mixture of dNTPs might be lethal to replicating viruses by virtue of the incorporation of uracil into replicating DNA.

cleoside diphosphate kinase (NDP kinase), which is also required for rNTP synthesis, phosphorylates all dNDPs to dNTPs. Because of its critical role in RNA synthesis, NDP kinase is always present and active within the cell. Therefore, the expression of ribonucleotide reductase alone, combined with the ever present NDP kinase activity, could lead to the synthesis of a potentially "toxic cocktail" of dNTPs (dUTP, dCTP, dATP, dGTP) in the cytoplasm of mammalian cells (Fig. 2A). It is important to note that the nuclear and mitochondrial localization of mammalian dUTPase, and the absence of this activity from the cytoplasm, would be critical to this scenario since this would allow dUTP to exist in the cytoplasm. We expect that such a mixture of dNTPs might be toxic to viruses attempting to replicate in the cytoplasm by promoting the synthesis of uracil-substituted viral DNAs. The subsequent attack on the viral DNA by a cellular uracil glycosylase may then lead to fragmentation of the viral genome or impair virus gene expression. Interestingly, human glyceraldehyde-3-phosphate dehydrogenase (G3PD), a cytoplasmic enzyme, also displays uracil glycosylase activity [31]. The reason why G3PD should have this additional enzymic function is unknown since the major nuclear and mitochondrial uracil glycosylases in humans are encoded by a different genes [32]. The model described here can explain why a *cytoplasmic* uracil glycosylase activity exists.

Clearly, this pattern of dNTP metabolism could represent a cellular defence mechanism directed against viral parasites that replicate in the cytoplasm (Fig. 2A) and would thereby account for the different distribution patterns of dUTPase and ribonucleotide reductase within a cell. If this notion is correct, it would seem that the existence of dUTPases among the B- and D-type retroviruses and the non-primate lentiviruses represents an adaptation to compromise such a defence system. Alternative strategies that viruses could adopt to avoid this system would be to link, in an obligatory way, viral DNA synthesis with cell proliferation, or to encode an inhibitor of the cellular uracil glycosylase enzyme. Interestingly, the HIV vpr protein has recently been found to bind the major cellular uracil glycosylase; however, it does not inhibit the activity of this enzyme [33].

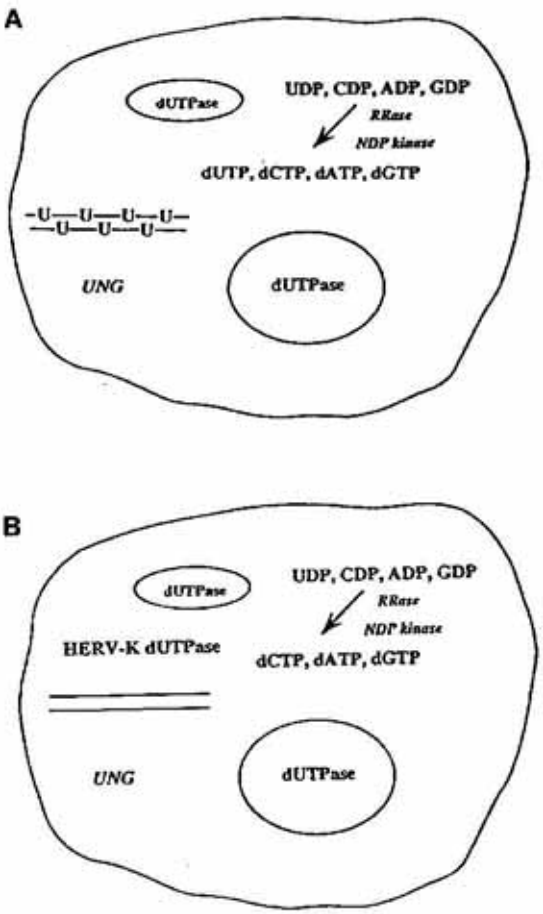

Fig. 2A. The "toxic cocktail" of dNTPs as a cellular defence mechanism against viral parasites.
The localization of the cellular dUTPase to the nucleus and mitochondria prevents uracil incorporation into DNA during replication in these organelles. Viruses replicating in the cytoplasm, however, are exposed to significant levels of dUTP resulting in uracil-substituted DNA. This interferes with viral gene expression, or provokes fragmentation of the viral genome through the action of uracil glycosylase-mediated excision repair. In humans, and perhaps other mammals, the cytoplasmic uracil glycosylase (UNG) may come from glyceraldehyde-3-phosphate dehydrogenase. Some viruses may have avoided this system by encoding their own dUTPases which are not localized to organelles. (RRase, ribonucleotide reductase).

2B. A possible role of the HERV-K (or HERV-L) dUTPase in HIV replication.
In the cells of old world primates, the fortuitous expression of a cytoplasmic dUTPase encoded by a human endogenous retrovirus may have circumvented the "toxic cocktail" defence by eliminating dUTP from this fraction of the cell. When lentiviruses were adapted for growth in these species, their own dUTPase gene was redundant in the presence of a HERV-encoded cytoplasmic enzyme. This removed selective pressure for the maintenance of this gene and thus it was rapidly deleted. Other mammalian lentiviruses (FIV, CAEV, EIAV) have retained a dUTPase gene presumably because their natural hosts lack a HERV dUTPase equivalent.

WHY DO PRIMATE LENTIVIRUSES LACK A dUTPase?

Of primary interest to us is the fact that, although *non-primate* lentiviruses such as CAEV, visna, EIAV and FIV contain dUTPase genes, the *primate* lentiviruses SIV and HIV do not. Based on the points raised above and other considerations described below, we suggest that this difference may have arisen evolutionarily from the existence of human endogenous retroviruses which encode dUTPases [34, 35]. The theory behind this hypothesis is outlined below.

Phylogenetic trees of lentivirus evolution, based on variations of reverse transcriptase genes, indicate that the primate lentiviruses are recent offshoots of their non-primate counterparts [36, 37]. This further implies that the common ancestor of the current lentivirus family contained a dUTPase gene and that the primate lentiviruses lost this gene during their adaptation for growth in primate hosts. This could have occurred if, for some reason, dUTP does not exist at a significant level in the cytoplasm of primate cells. However, at one point in time, dUTP levels likely represented a problem for retroviruses of old world primates. This is evident from the fact that some human endogenous retroviruses encode dUTPase enzymes. The human endogenous retrovirus K (HERV-K) is a vertically transmitted, defective retrovirus that entered the germ line of old world primate ancestors 33–40 million years ago [38] subsequent to the divergence of old and new world species and prior to the appearance of the hominoids. It has been estimated that there are 50–100 copies of the provirus per diploid cell [34]. Amino-acid sequence comparisons have established that this virus clearly contains a dUTPase gene [21], which is evidence that dUTP must have been present at significant levels in the cytoplasm of old world primate ancestors.

If dUTP levels were a problem for primate retroviruses then, why not now? One possible explanation (among others not discussed here) is that a HERV-K encoded dUTPase may be expressed fortuitously in many old world primates. This is a distinct possibility, given the large copy number of the HERV-K provirus and its random distribution within the human genome. Since the proviral enzyme would lack nuclear or mitochondrial targeting signals, it would be expected to reside in the cytoplasm and, if active, eliminate dUTP from this region of the cell (Fig. 2B). This would effectively remove the "toxic cocktail" of dNTPs as a barrier to further virus challenges. When lentiviruses subsequently adapted from non-primate to primate hosts, their dUTPase gene would have been redundant in the presence of the cytoplasmic HERV-K encoded enzyme. In turn, this would have eliminated the selective pressure for the maintenance of a dUTPase gene and thereby facilitated its rapid deletion from the primate progenitor of HIVs and SIVs. Since the HERV-K provirus is common to old world primate species, this scheme of events could account for the fact that the HIV and SIV viruses, as well as the human oncoretroviruses HTLV-I and HTLV-II, do not encode dUTPases. *On the other hand, non-primate species may lack HERV analogues, which would account for the fact that current non-primate lentiviruses encode dUTPases.* Interestingly, a retrovirus of squirrel monkeys, which are new world primates, contains a dUTPase gene [21]. This is consistent with the evolutionary pattern described above, since these primates do not contain a HERV-K provirus.

Although it is not yet known whether a functional HERV-K dUTPase is expressed in human cells, full-length and processed HERV-K transcripts and also various HERV-K antigens have been detected in human cell lines, including the virus protease which contains the dUTPase functional domain [39–41]. We have purified a recombinant form of the HERV-K dUTPase and find it to have activity comparable to that of the MMTV enzyme (J. Harris, E.M. McIntosh & R.H. Haynes, unpublished results). Thus, it is possible that a HERV-K dUTPase is expressed in human cells and we are currently investigating this question. In addition to HERV-K, Cordonnier *et al.* [35] have recently characterized a new class of endogenous retrovirus, HERV-L, which also encodes a dUTPase. This virus, which is phylogenetically more similar to human foamy viruses than other HERVs, is

present at 100–200 copies per cell. Although the expression of this HERV in human cells has yet to be determined, potentially it could supply an alternative source of cytoplasmic dUTPase and therefore fulfil the requirements of the model described above. Obviously, we cannot rule out the possibility that other proviral sources of dUTPase may also exist in human cells.

We conclude that the survival of HIV in human macrophages may be largely dependent upon genetic complementation by the HERV-K or HERV-L encoded dUTPase. Furthermore, it is of interest to note that HIV-positive individuals display markedly enhanced titres of antibodies to HERV-K antigens [41]. Thus, the possibility also exists that HIV infection of macrophages can induce expression of HERV-K genes such as the dUTPase-encoding *gag*. Conversely, it is possible that there are individuals within the population who do not express HERV-K genes under any circumstances and may therefore be completely refactory to productive HIV infection.

HERV-K dUTPase — A POTENTIAL TARGET FOR DRUG DEVELOPMENT?

If the HERV-K (or HERV-L) dUTPase is necessary for efficient HIV replication, it may provide another possible target for anti-HIV drug development. One advantage of considering this enzyme as a target is that it is unlikely to play any important role in cellular metabolism and therefore inhibitors would not be expected to be cytotoxic. (This would not necessarily be the case if the dUTPase required for HIV replication is derived by "leakage" from the nuclear and/or mitochondrial pools of the "native" human isoforms that are found in these organellar compartments). Such inhibitors should block HIV replication by restoring a cytoplasmic dUTP pool and thereby the "toxic cocktail" of dNTPs. As suggested above, an additional advantage of the HERV-K dUTPase as a target is that it is not encoded by HIV and therefore resistance to inhibitors would be independent of HIV mutation rates. Two approaches can be taken in this respect. First is the identification or design of deoxyuridine or other nucleoside analogues or compounds that inhibit the HERV dUTPase without affecting the cellular enzyme. This is feasible since (i) there is only a low degree of amino-acid sequence identity (24%) between the HERV-K and human dUTPases, (ii) both enzymes have been expressed and purified in high yield from *E. coli*, and (iii) the crystal structure of the human enzyme complexed with substrate has recently been determined [42]. Thus, crystallization of the HERV-K enzyme may allow for modelling and design of analogues or other inhibitory compounds. A second possible approach would be to use antisense oligonucleotides to block HERV-K gene expression. HERV-L, or other proviral dUTPases (should they exist) could be attacked in a similar way.

Prior to such efforts, however, it will first be necessary to establish whether a functional HERV dUTPase is indeed expressed in the cytoplasm of human cells. This can best be addressed through immunological approaches. We are currently pursuing this work.

SUMMARY

It is now evident that dUTP is a toxic metabolite by virtue of its ability to be utilized by DNA polymerases. The currently available data for cellular systems show that the incorporation of uracil into DNA leads to cell death owing to the action of uracil glycosylase-mediated excision repair. The fact that a variety of mammalian retroviruses encode dUTPases is evidence that dUTP exists at significant levels in the cytoplasm of at least some types of mammalian cells. Furthermore, the existence of endogenous retroviruses carrying dUTPase genes argues that cellular dUTP pools have represented a problem for virus replication in mammalian cells for millions of years. We propose that the relative subcellular distribution of ribonucleotide reductase and dUTPase facilitates the synthesis of a toxic cocktail of cytoplasmic dNTPs which acts as a metabolic poison for parasitic viruses. This protective mechanism has been compromised in primates by the expression of an endogenous retrovirus dUTPase that is not subject to differential subcellular distribution, and has allowed the evolution of HIVs and SIVs lacking a dUTPase gene. Thus, we suggest that

the survival of HIV in human macrophages may be largely dependent upon the fortuitous expression of a HERV-encoded dUTPase. If this hypothesis is correct, it implies that inhibitors of the proviral enzyme (e.g. deoxyuridine analogues) or its synthesis (e.g. antisense oligonucleotides) may be useful for HIV therapy. Furthermore, it points to an unusual aspect of cellular metabolism, the purpose of which is to act as a defensive mechanism against viral parasites.

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