Extensive editing of a small fraction of human T-cell leukemia virus type 1 genomes by four APOBEC3 cytidine deaminases

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In the absence of the human immunodeficiency virus type 1 (HIV-1) Vif protein, the host-cell cytidine deaminases APOBEC3F and -3G are co-packaged along with virion RNA. Upon infection of target cells, nascent single-stranded DNA can be edited extensively, invariably giving rise to defective genomes called G→A hypermutants. Although human T-cell leukemia virus type 1 (HTLV-1) replicates in the same cell type as HIV-1, it was shown here that HTLV-1 is relatively resistant to the antiviral effects mediated by human APOBEC3B, -3C, -3F and -3G. Nonetheless, a small percentage of genomes (0·1 < f < 5 %) were edited extensively: up to 97 % of cytidine targets were deaminated. In contrast, hypermutated HTLV-1 genomes were not identified in peripheral blood mononuclear cell DNA from ten patients with non-malignant HTLV-1 infection. Thus, although HTLV-1 DNA can indeed be edited by at least four APOBEC3 cytidine deaminases in vitro, they are conspicuously absent in vivo.

Supplementary figures are available in JGV Online.

Human immunodeficiency virus (HIV) cDNA synthesis is exquisitely sensitive to editing by the host-cell cytidine deaminases APOBEC3F and -3G, to the point that the virus encodes a gene whose product, the Vif protein, neutralizes the effect of these single-stranded DNA deaminases (Harris et al., 2003; Lecossier et al., 2003; Mangeat et al., 2003; Mariani et al., 2003; Wiegand et al., 2004; Zhang et al., 2003; Zheng et al., 2004). On an HIV-1 Δvif background, APOBEC3F and -3G molecules may be co-packaged into the virion (Wiegand et al., 2004; Zheng et al., 2004). Following infection of a target cell, synthesis of minus-strand DNA occurs, which is deaminated immediately by the APOBEC3 molecules within the reverse-transcription complex (Beale et al., 2004; Bishop et al., 2004; Harris et al., 2003; Lecossier et al., 2003; Liddament et al., 2004; Mangeat et al., 2003; Mariani et al., 2003; Susséne et al., 2004; Wiegand et al., 2004; Yu et al., 2004b; Zhang et al., 2003; Zheng et al., 2004). Such heavily edited genomes are referred to as G→A hypermutants, as cytidine deamination on the minus strand (C→U) shows up as G→A transitions on the reference plus strand. G→A hypermutants have been described for most of the lentiviruses (Fitzgibbon et al., 1993; Gao et al., 1992; Perry et al., 1992; Vartanian et al., 1991; Wain-Hobson et al., 1995), of which HIV-1 is the most prominent member. The situation is strikingly different for another human retrovirus, human T-cell leukemia virus type 1 (HTLV-1). Despite a considerable sequence database, there has only been one naturally occurring G→A hypermutated HTLV-1 sequence reported to date (Vartanian et al., 1997). It is worth noting that this sequence was derived from an animal model, i.e. experimental HTLV-1 infection of a squirrel monkey (Saimiri sciureus). Given that APOBEC3G from different primates restricts both HIV and simian immune-deficiency virus in a variable manner (Ribeiro et al., 2005; Yu et al., 2004a), it has not been formally demonstrated that human APOBEC3 molecules can restrict HTLV-1 in vivo. In addition, G→A hypermutants are rarely found in analyses of HTLV-1 reverse-transcription errors (Mansky, 2000). It is intriguing that HTLV-1 is apparently resistant to the effects of APOBEC3 molecules, given that it replicates in the same cell type as HIV in vivo, the CD4+ T lymphocyte. To address the dichotomy, a phenotypic assay was used to study the effects of APOBEC3 molecules on HTLV-1 infection. In addition, a highly sensitive PCR-based protocol was used, referred to as 3DPCR, that is capable of amplifying G→A hypermutated genomes (Susséne et al., 2005a). This PCR method relies on the fact that the DNA of an AT-rich variant melts at a slightly lower temperature than the parental DNA. By lowering the denaturation temperature, it is possible to selectively amplify G→A hypermutants. In an experimental setting, it was shown here that hypermutants occur at frequencies within the range 0·1 < f < 5 %.

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To explore the effects of APOBEC3 molecules on HTLV-1 infectivity, human 239T cells were transfected with DNA from an infectious molecular clone of HTLV-1 (pCS-HTLV-1; Derse et al., 1995) in the presence and absence of four different human APOBEC3 gene-expression plasmids, namely APOBEC3B, -3C, -3F and -3G. Twenty-four hours post-transfection, cells were co-cultivated 1:1 with susceptible 293T–HTLV-1–LTR–GFP indicator cells (Delebecque et al., 2002). Two days later, cells were collected and 10⁵ cells were analysed by flow cytometry for green fluorescent protein (GFP) expression as described previously (Royer-Leveau et al., 2002). These cells have the GFP gene expressed under the control of the HTLV-1 Tax-dependent long terminal repeat. Infection by HTLV-1 results in early expression of the viral Tax protein and hence GFP. The percentage of GFP-positive indicator cells was determined by flow cytometry (Fig. 1a). A Kruskall–Wallis non-parametric test showed no statistically significant differences between any of the five categories with HTLV-1 (χ² = 8.75, P = 0.068).

We recently reported a novel PCR protocol that allows differential amplification of G→A hypermutants (Suspené et al., 2005a). Based on PCR, it can pick up relatively rare events that might not be scored by the phenotypic assay (Fig. 1a). The first round of PCR was performed at 95 °C using the primer pair P1 (5′-ARGCCGCCATCCACGCGRRTT-3′) and P2 (5′-TYTTGAGATGTYTATAGAAT-3′), where R=A/G and Y=T/C. The minimal denaturation temperature (Tₐ) required to amplify the parental HTLV-1 locus in the R–U5 region was found to be 93 °C. First-round reaction parameters were 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, with final extension for 10 min at 72 °C. A selective amplification of hypermutants was performed in the second round, using the equivalent of 0.5 μl of the first-round reaction as input with the primer pair P2 and P3 (5′-CCTRAACTACRTGCCRTCT-3′) at a denaturing temperature of 91 °C (Tₐ = 2 °C), which yielded a 248 bp fragment. Cycling conditions were 91 °C for 5 min, followed by 35 cycles of 91 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, with final extension for 10 min at 72 °C. HTLV-1 DNA was recovered from all four co-transfections, i.e. APOBEC3B, -3C, -3F and -3G, but was not recovered from cells transfected by the parental pCS-HTLV-1 plasmid alone (Fig. 1b). The specificity of 3DPCR is shown in Fig. 1(c) where the second-round PCR was performed at Tₐ = 95 °C. As expected, DNA products were obtained in all cases.

3DPCR products were purified from agarose, cloned and up to 50 clones were sequenced. A representative selection of six molecular clones from each co-transfection is shown in Fig. 2, while the full sequence sets are given in Supplementary Fig. S1 (available in JGV Online). All sequences but one (see below) bore the classical traits of retroviral G→A hypermutants – intense and monotonous substitution of G for A and dinucleotide context preferences. The number of transitions ranged from 6 to 48 per clone, which translated into G→A substitution frequencies of between 10 and 79 %.

Limiting dilution of the second-round hypermutated PCR products (i.e. those amplified at 91 °C) showed that they titrated out to a frequency of ~10⁻⁵ for all four HTLV/APOBEC3 co-transfection experiments (data not shown). By contrast, limiting dilution of second-round products amplified at 95 °C titrated out to ~10⁻⁸. Given that there must be residual plasmid DNA in the samples, the proportion of edited HTLV-1 genomes must be >10⁻³.

Electrophoresis of first-round products in HA-yellow (Hanse Analytik), a pegylated bisbenzamide that interacts preferentially with the minor groove of AT-rich DNA, thus retarding migration (Janini et al., 2001; Suspené et al., 2005a), failed to identify any band shift to high molecular mass, which would be indicative of AT-rich genomes. Furthermore, analysis of cloned first-round products from the APOBEC3G co-transfection failed to identify any edited

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**Fig. 1.** Effect of APOBEC3 proteins on HTLV-1 infectivity. (a) The y axis indicates the percentage of GFP-positive cells following co-culture with pCS-HTLV-1-transfected cells. The box-whiskers plots represent the maximum, first-quartile, median, third-quartile and minimum values derived from four transfections. Cells (100 000) were analysed by flow cytometry. C, Negative transfection control; wt, pCS-HTLV-1 molecular clone alone; 3B, 3C, 3F and 3G, individual co-transfections with the various APOBEC3 expression plasmids. (b) Second-round PCR products amplified selectively by using Tₐ = 91 °C. (c) Second-round PCR products amplified by using Tₐ = 95 °C as controls for (b).
Among the 48 sequences derived from the APOBEC3G/HTLV-1 co-transfection, one hypermutant actually encoded six C→T transitions and a single G→A transition (sequence R1, Supplementary Fig. S1). Although rare, this is not without precedent for HIV-1 (Vartanian et al., 1991) and has been observed more recently for hepatitis B virus (Suspe`ne et al., 2005b). As APOBEC3G is not an RNA cytidine deaminase (Harris et al., 2003; Lecossier et al., 2003; Mangeat et al., 2003; Mariani et al., 2003; Suspe`ne et al., 2004; Zhang et al., 2003), one interpretation is that during the final stages of plus-strand DNA synthesis, strand displacement resulted in a single-stranded R–U5 DNA segment susceptible to cytidine deamination.

3DPCR was also performed at 87 °C (Td−6 °C), the lowest denaturation temperature at which HTLV-1 DNA could be recovered. Sequencing of cloned products showed that the sequences were far more homogeneous than those amplified at 91 °C. In the case of APOBEC3C, 17/20 clones sequenced were identical (data not shown). We have shown previously that the lower the denaturation temperature, the more hypermutated the molecules recovered (Suspe`ne et al., 2005a). Presumably, the proportion of hypermutated HTLV-1 genomes denatured at 87 °C represented a subset of those that melted at 91 °C, resulting in greater internal homogeneity. In keeping with this, the mean substitution frequencies of these hypermutants ranged from 79 to 97 %, which was generally higher than those reported in Figs 2 and 3.
S1, i.e. 10–79%. These findings caution against using the lowest possible PCR denaturation temperature to amplify hypermutants, as it will result in a biased and unrepresentative collection of sequences.

Given the extraordinary ability of 3DPCR to selectively amplify G→A-hypermutated genomes, it was used to screen HTLV-1-positive patient material. High-molecular-mass DNA was extracted from peripheral blood mononuclear cells (PBMCs) of ten patients with non-malignant HTLV-1 infection. There were two males and eight females of mean age 49 years (range 29–56 years). Two were asymptomatic carriers and seven presented with tropical spastic paraparesis/HTLV-1-associated myelopathy (TSP/HAM), one with HTLV-associated myelitis. Samples from patients with adult T-cell leukaemia were not studied, because the DNA proviral load results from a few cells that have undergone massive clonal expansion. Selective PCR was performed on the same R–U5 locus analysed above at 91 °C. Products were recovered from three samples (one asymptomatic carrier and two with TSP/HAM). All samples were shown to be positive when amplified at 95 °C, which served as positive control for DNA quality. However, upon sequencing of cloned 3DPCR products, it was apparent that the genomes represented a collection of AT-rich variants and not bona fide G→A hypermutants. This followed from inspection of the sequences, as well as the symmetry in C→T and G→A transitions in the mutation matrices (see Supplementary Figs S2 and S3, available in JGV Online). Such a finding is not without precedent for 3DPCR-recovered AT-rich genomes from a poliovirus mutant spectrum (Suspené et al., 2005a). As the method can identify G→A-hypermutated genomes at frequencies of 10⁻⁴ (Suspené et al., 2005b), the inability to identify such genomes in patient material demonstrated that HTLV-1 G→A hypermutants remain extremely rare in vivo.

The present data showed that, in cell culture, APOBEC3-edited minus-strand HTLV-1 DNA is a rare phenomenon. However, when editing occurs, the substitution frequency can reach 97%. As in vitro work has shown a dose–response relationship between APOBEC3G concentration and deamination (Suspené et al., 2005b), extensively deaminated genomes imply large numbers of APOBEC3 molecules per virion. It might be argued that, like HIV-1, HTLV-1 encodes a gene product protecting it from the adverse effects of APOBEC3 molecules. However, it should be noted that the HTLV-1-bearing MT2 T-cell line is non-permissive for a HIV-1 Δvif virus, an observation that is not consistent with this hypothesis (Sova & Volsky, 1993).

HIV and HTLV-1 preferentially infect the same CD4⁺ T lymphocytes in vivo, and clearly both viral genomes are formally prone to hypermutation. By contrast, HIV-1 G→A hypermutants are far more numerous than their HTLV-1 counterparts in vivo. How can these findings be reconciled? One variable could be the mode of replication of the two viruses. In vivo, HIV goes through ~200 rounds of productive replication year⁻¹ (Ho et al., 1995; Pelletier et al., 1995; Perelson et al., 1996; Wei et al., 1995), whereas HTLV-1 replicates via Tax-driven clonal expansion of the host cell (Wattel et al., 1995) and only infrequently via reverse transcription. This means that, for a given DNA copy number, there are fewer occasions for APOBEC3 editing of nascent HTLV-1 minus-strand DNA as opposed to HIV-1 DNA.

The in vitro experimental data are, however, far less vulnerable to negative selection by clonal expansion, as DNA was recovered 2 days after co-cultivation. The fact that most of the hypermutated genomes were unique indicates that 3DPCR was amplifying multiple independent genomes. One possibility is that HTLV-1 and HIV-1 bud from subtly different regions of the plasma membrane where the

![Graph showing substitution frequency as a function of the 5’ nucleotide.](image)

*Fig. 3.* Dinucleotide context associated with APOBEC3 editing. The y axis represents the percentage substitution frequency as a function of the 5’ nucleotide. Dots indicate the deaminated C residue in the minus strand. The expected value is represented by a horizontal bar and corresponds to the base composition of the locus. Differences between the observed and expected values were assessed by using \( \chi^2 \) analysis. Statistically significant deviations are indicated by an asterisk (\( P<0.001 \)).
concentrations of APOBEC3 molecules differ considerably. An alternative could be that the expression of APOBEC3 molecules, and hence editing, is out of phase in the cell cycle with HTLV-1 assembly, whereas that of HIV-1 is in phase. In either hypothesis, to achieve a small fraction of extensively hypermutated HTLV-1 genomes requires just a small overlap with the mechanism by which HIV-1 genomes are edited.

In conclusion, the present observations demonstrate that human APOBEC3B, -3C, -3G and -3F restriction of HTLV-1 replication formally parallels the HIV paradigm more closely than realized previously. However, APOBEC3 editing of HTLV-1 DNA remains a rare phenomenon in vitro and is so far undetectable in vivo.

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