HIV infection *en route* to endogenization: two cases

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Abstract

The long-term spontaneous evolution of humans and the human immunodeficiency virus (HIV) is not well characterized; many vertebrate species, including humans, exhibit remnants of other retroviruses in their genomes that question such possible endogenization of HIV. We investigated two HIV-infected patients with no HIV-related disease and no detection with routine tests of plasma HIV RNA or cell-associated HIV DNA. We used Sanger and deep sequencing to retrieve HIV DNA sequences integrated in the human genome and tested the host humoral and cellular immune responses. We noticed that viruses from both patients were inactivated by the high prevalence of the transformation of tryptophan codons into stop codons (25% overall (3–100% per gene) and 24% overall (0–50% per gene)). In contrast, the humoral and/or cellular responses were strong for one patient and moderate for the other, indicating that a productive infection occurred at one stage of the infection. We speculate that the stimulation of APOBEC, the enzyme group that exchanges G for A in viral nucleic acids and is usually inhibited by the HIV protein Vif, has been amplified and made effective from the initial stage of the infection. Furthermore, we propose that a cure for HIV may occur through HIV endogenization in humans, as observed for many other retroviruses in mammals, rather than clearance of all traces of HIV from human cells, which defines viral eradication.

Keywords: APOBEC, cure, DNA integration, endogenization, endogenous retrovirus, human immunodeficiency virus, stop codon, tryptophan, Vif

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Introduction

Human immunodeficiency virus (HIV) is one of the three 'big killers' and causes ≈35 million infections worldwide [1]. Despite massive efforts, attempts to cure people infected with this virus have failed [2,3], except for an adult HIV-positive patient who exhibited long-term aviraemia without antiretroviral therapy following transplantation of stem cells from a donor carrying the protective CCR5 Delta32/Delta32 deletion [4]. This failure to reach a cure for HIV was described as primarily due to the absence of eradication of any trace of replication-competent HIV DNA integrated into human cell genomes [3].

Since the beginning of the HIV pandemic, 5–15% of HIV-infected individuals have been identified as devoid of clinical symptoms and progression in the absence of antiret-

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roviral treatment [5,6]. Some of them, representing <1% of HIV-positive persons, exhibit spontaneous control of HIV replication, including the so-called ‘elite controllers’ (ECs), who spontaneously and durably exhibit undetectable or almost undetectable virus in plasma and a normal CD4-cell count [6,7]. ECs attracted attention and sparked research to identify the factors associated with spontaneous functional cure of HIV infection. Various potential mechanisms involving viral, host genetic and immunological patterns were identified, but none of them could explain the spontaneous control of HIV replication alone or in all of the cases [5–8].

We investigated here a patient who had been HIV-seropositive since 1985 with asymptomatic infection in the absence of antiretroviral treatment and no detectable infectious virus nor any HIV RNA or DNA genomic material retrieved from the plasma or peripheral blood mononuclear cells (PBMCs), and who thus appeared to be cured. We studied in depth his host response and searched for any trace of HIV DNA integrated into PBMCs. In addition, we searched for other similar cases in our series.

Methods

Patients

Our patient cohort was composed of 1700 HIV-infected patients, including ten elite HIV-1 controllers. Among these ten, two also had undetectable PBMC HIV DNA; they gave their written informed consent to be included in the study.

Host testing

Anti-HIV-1 antibody testing was performed using an enzyme-linked immunosorbent assay (Architect; Abbott Diagnostics, Mannheim, Germany) and Western blot testing (Bio-Rad, Stanford, CA, USA). Analysis of plasma tryptophan (W) levels was performed using the 7300 High Performance Amino Acid Analyzer (Beckman Instruments, Inc., Fullerton, CA, USA). APOBEC mRNA quantification in the PBMCs was performed. The sequence of gene encoding the CCR5 chemokine receptor and HLA genotype were determined. Resistance of the PBMCs to HIV super infection was assessed by inoculating these PBMCs with HIV-1 strain NL4.3. To test the inhibitory effect of the two case-patients’ sera on HIV growth, the NL4.3 strain was cultured on control PBMCs in the presence of 100 μL of these sera or control sera (see Supporting information).

Immunological investigations

For flow cytometry analyses, PBMCs were stained with the following monoclonal anti-human antibodies used in various combinations: CD3, CD4, CD38, HLADR, CCR7, granzyme B, perforin, TNFα, IFNγ and MIP1β (BD Biosciences, San Jose, CA, USA); CD8 and CD45 RA (eBioscience, San Diego, CA, USA). To study HIV-specific T-cell responses, PBMCs were cultured in the presence of HIV-specific antigens consisting of a mix of 15-mers overlapping 11-amino acid peptides (36 peptides in Gag, Pol and Nef sequences). On day 2, 100 U/mL recombinant IL-2 (Miltenyi Biotec, Bergisch Gladbach, Germany) was added, then cells were harvested on day 7, and re-stimulated with the same pool of peptides for 6 hours in the presence of anti-CD28/anti-CD49d co-stimulatory antibodies and Golgi Plug (BD Biosciences). Thereafter, cells were stained for intracytoplasmic cytokines (TNFα, IFNγ and MIP1β) (see Supporting information).

Virological investigation

Culture assays. Culture assays were performed as described previously [9] and in the Supporting information.

HIV nucleic acid and protein detection. Routine plasma HIV RNA testing was performed using the RealTime HIV-1 assay (Abbott Diagnostics) and the Generic HIV-RNA assay (Biocentric, Bandol, France) (detection limit, 40 and 300 copies/mL, respectively). Total (integrated and unintegrated) cell-associated HIV DNA testing was performed as previously described (detection limit, 20 copies/10^6 PBMCs) [10]. HIV protein identification in serum was performed by immunoprecipitation with anti-HIV P24 monoclonal mouse antibodies using magnetic beads coupled with protein A (Dynabeads Technology, Invitrogen, Carlsbad, CA, USA). The precipitate was separated by SDS-PAGE and analysed using MALDI-TOF mass spectrometry and a search in viral protein databases (see Supporting information).

HIV genome sequencing and analyses. HIV genome sequencing was performed using Sanger population and next-generation sequencing of HIV-1 DNA from the two case-patients’ PBMCs and, to obtain missing fragments of the HIV genome, a new procedure named the ‘Bortsch’ procedure. HIV sequences obtained from the two case-patients are available from GenBank with accession numbers KM878756-810 and KM878811-833. Phylogenetic analyses and subtyping are described in the Supporting information. HIV genomes from ECs and non-ECs were searched for in GenBank (http://www.ncbi.nlm.nih.gov/nuccore/), and retrieved genomes were compared with those from the two case-patients, seeking stop codons, hypermutations, insertions/deletions and other mutations in each HIV-1 protein sequence. For comparison of the W-to-stop mutations in ECs and non-ECs, hypermutated genomes from the Eyzaguirre *et al.*

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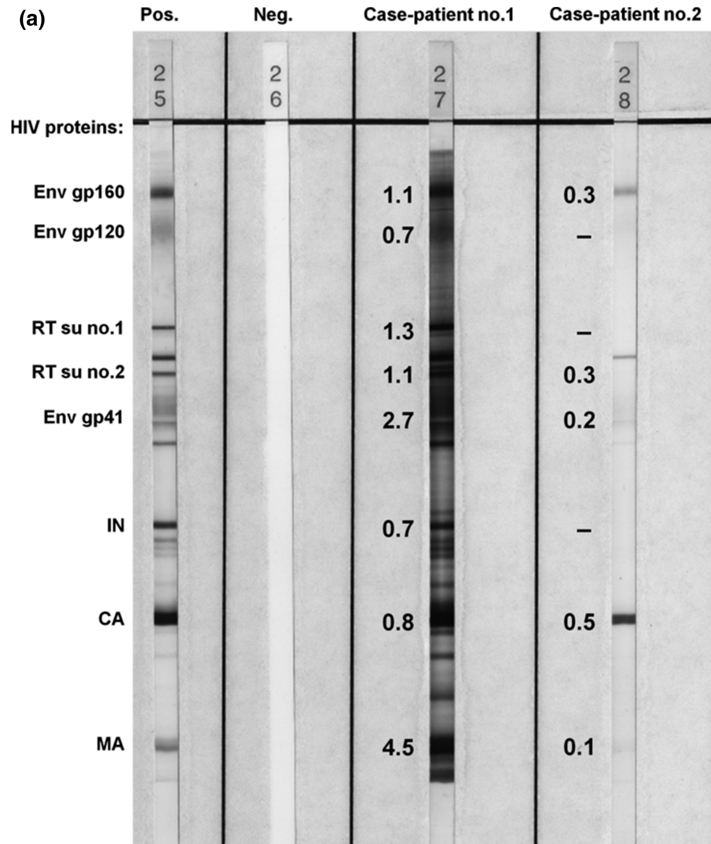

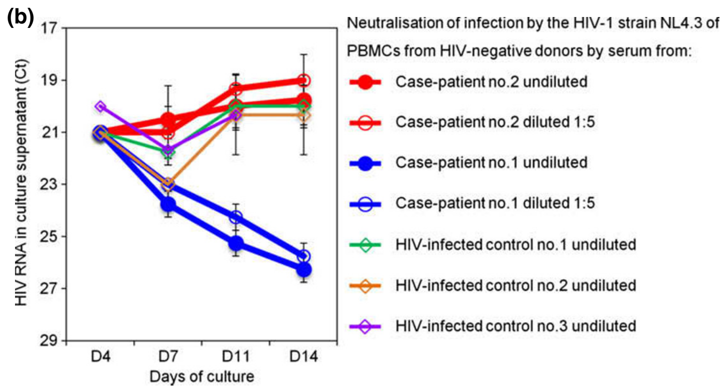

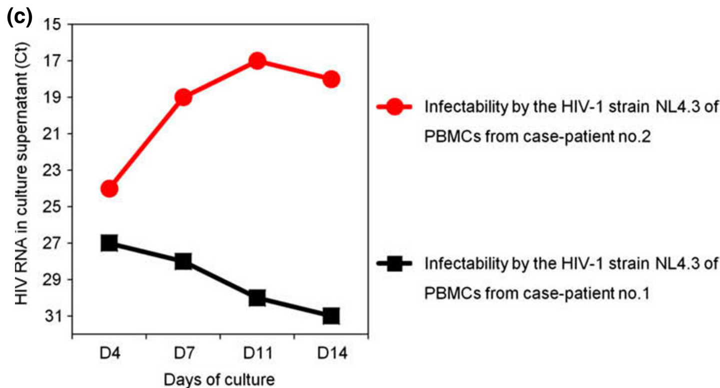

FIG. 1. (a) Western blot HIV-1 for serum samples from the two case-patients. Values near the Western blot indicate the ratio between the signal obtained for the band compared to that from the positive control (see Supporting information). CA, capside; Env, envelope; gp, glycoprotein; IN, integrase; MA, matrix; Pos., positive control; Neg., negative control; su, subunit; RT, reverse transcriptase. (b) Seroneutralization by the case-patients' serum of infection of peripheral blood mononuclear cells from HIV-negative donors by HIV-1 strain NL4.3. Ct, PCR cycle threshold; PBMCs, peripheral blood mononuclear cells. (c) Infectability of peripheral blood mononuclear cells from case-patients by the HIV-1 strain NL4.3. Ct, PCR cycle threshold; PBMCs, peripheral blood mononuclear cells.

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study [11] were used, the sequence of each gene being translated then aligned to detect stop codons (see Supporting information).

Results

The index case is a 57-year-old patient who heavily used intravenous drugs between 1978 and 1988. He was diagnosed HIV-positive in 1985 and did not suffer from any clinical manifestations linked to HIV infection. He claimed to exhibit a special immunity, presenting no fever or lymphadenopathy during his life. His CD4-cell count was always normal (CD4- and CD8-cell counts were 1576 and 1234, respectively) and no HIV RNA was ever found in his plasma nor HIV DNA in his PBMCs. In contrast, he suffered from active hepatitis C infection. He was continuously exposed to HIV as his wife shared syringes with him until 1988; she was diagnosed HIV-positive in 1985 and developed AIDS with a different virus (see Supporting information). This patient's resistance to HIV justified an in-depth investigation of both his host response and his integrated retrovirus.

Strong reactivity against all tagged proteins was noted on the Western blot (Fig. 1a). No homozygosity or heterozygosity for the 32-base pair (bp) deletion in the CCR5 co-receptor gene was found. HLA genotype is not associated with HIV protection. The serum of the patient was found to protect HIV-negative PBMCs from the virulent HIV-1 strain NL4.3 (Fig. 1b and Supporting information). This was also observed for three out of eight sera from HIV-positive controls but not for sera from the HIV-negative controls, indicating that NL4-3 can be neutralized by some HIV-positive sera [12]. Moreover, an attempt to obtain an infection of the PBMCs from this patient with the same virulent strain was unsuccessful (Fig. 1c), although this does not allow speculation on susceptibility to other HIV strains. We conclude that this patient was immune to super infection with HIV. We were unable to grow HIV from this patient but we detected in his serum three viral peptides with similarities to HIV-1 reverse transcriptase (see Supporting information). Sequencing the HIV DNA integrated into the PBMCs of the patient was extremely laborious because of the very low copy number (<20 copies/10^6 PBMCs), but we obtained a 9302-bp-long subtype B HIV genomic sequence using Sanger population, clonal sequencing and next-generation sequencing (Fig. 2) (see Supporting information). Surprisingly, we found many stop codons, all at W codon (TGG) positions, reaching 25% of the 92 W codons of the whole genome and 100% in three HIV genes (Fig. 2, Table 1 and Supporting information, Table S1). Therefore, we concluded that the virus was inactivated and the patient was cured.

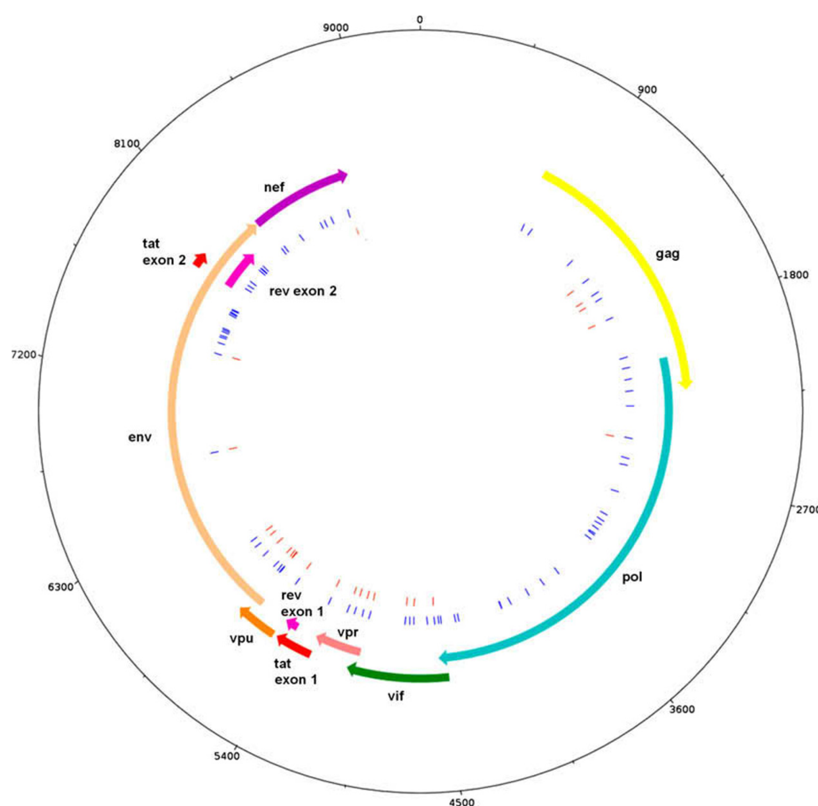

FIG. 2. HIV genome retrieved from case-patient no. 1. HIV genes are shown on the outer ring. On the inner rings, blue lines indicate tryptophan (W) codons in genes; red lines indicate W-to-stop mutations. Representation was built using DNAplotter (http://www.sanger.ac.uk/Software/Artemis/circular/).

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TABLE 1. Distribution of tryptophan (W)-to-stop mutations in HIV genomes from the two case-patients and natural viral suppressors, at positions devoid of such mutations in two sets of control HIV-infected patients, one on highly active antiretroviral therapy and the other untreated

	HIV genes	gag		pol						vif			vpr	vpu	env	nef	
	Codons	gag36	gag265	pol62	pol179	pol394	pol776	pol823	pol958	vif21	vif79	vif89	vpr38	vpu23	env840	nef57	nef183
Proportion of sequences harbouring a W-to-stop mutation for case-patients (%)	Case patient no.1	0	17	0	25	0	0	0	0	16	0	22	33	79	0	0	17
	Case patient no.2	0	22	0	25	25	0	0	0	0	0	0	0	0	17	0	0
Number of sequences harbouring a W-to-stop mutation for natural viral suppressors ($n = 23$)		4	6	1	1	2	1	1	4	4	1	2	0	6	0	1	3

HIV genomes analyzed here are from the article by Eyzaguirre *et al.*[11]

The second patient in our cohort was also considered to be an HIV EC with no detectable RNA or DNA using standard methods. This 23-year-old homosexual Chilean man was identified as HIV-infected in 2011 and most likely infected in Chile during the 3 previous years, and his HIV sequence was clustered with South American viral genomes (see Supporting information). His serum showed weak anti-HIV reactivities on the Western blot (Fig. 1a). T-cell phenotypic characteristics of the patient, including frequency of different T-cell subpopulations and the activation or cytotoxic marker expression by CD4 and CD8 T-cells, did not differ from those of an HIV-infected control patient (Fig. 3a). Despite a low CD8+ T-cell response, this patient exhibited high CD4+ T-cell responses in terms of cytokine production (42.8%, 37.9% and 40.1% of CD4+ T-cell-producing IFN-γ, TNF-α and MIP1β, respectively) compared with a group of 22 HIV controls (mean $\pm$ standard deviation (SD) = 5.5 $\pm$ 14.4%, 4.5 $\pm$ 10.2% and 5.9 $\pm$ 15.5%) (Y. Levy, personal data) (Fig. 3b). Serum from this patient did not inhibit HIV infection, and his PBMCs were susceptible to infection by the NL4.3 strain (Fig. 1b-c; see Supporting information). This patient did not harbour homozygosity/heterozygosity for the 32-bp deletion in the CCR5 co-receptor gene and his HLA genotype is not HIV protective. HIV co-culture was unsuccessful. HIV genome sequencing from the patient's PBMCs showed that he exhibited 21 stop codons, with all but two being located at W codons (Tables 1 and S1).

When comparing the distribution of stop codons replacing W, in case-patient 1, the accessory and regulatory genes *vif*, *vpr*, *vpu* and *tat* were mostly affected ($\geq$50 to 100% of W-to-stop mutations in these genes), whereas in case-patient 2, only the *gag*, *pol*, *env* and *nef* genes were affected (Supporting information, Table S1). We then compared the HIV genomes from ECs and non-ECs. The median rate of W-to-stop mutations was significantly higher in ECs (including the two present cases) than in non-ECs (20 vs. 9; $p < 0.001$; Mann-Whitney test) (Supporting information and Table S1). In addition, this mutation was present at 16 particular W codons only in the ECs, and at codons 38 in *vpr* and 840 in *env* only in our two case-patients.

Discussion

The two case-patients studied here are apparently cured of their HIV infection and one exhibits cells resistant to HIV infection *in vitro*. Functional cure, as defined by spontaneous control of HIV infection without disease progression, was therefore achieved [13]. This occurred despite HIV DNA sequences could be laboriously retrieved from PBMCs.

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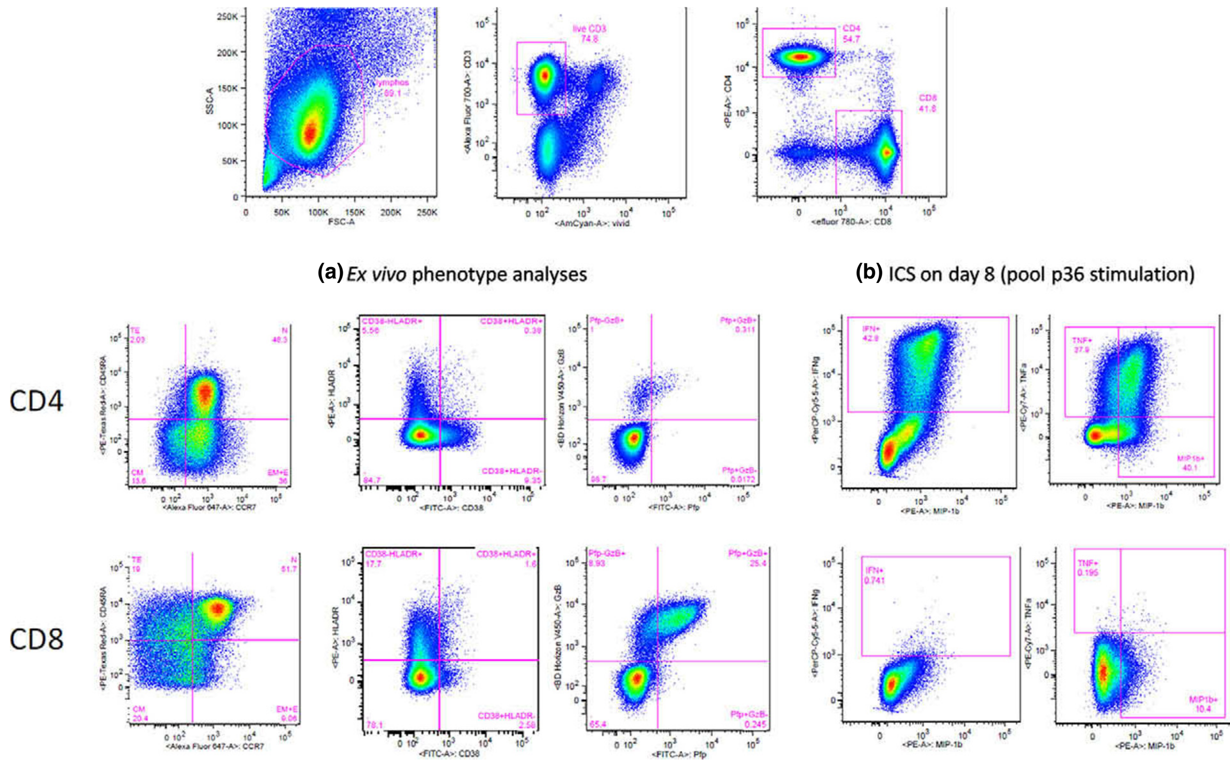

FIG. 3. Flow cytometric profiles of T cells of case no. 2. (a) *Ex vivo* phenotype of T cells. Differentiation (CD45RA, CCR7), activation (CD38, HLADR) and cytotoxic (Granzyme B, Perforin) markers were used to analyse CD4 (upper panel) and CD8 (lower panel) T cells. (b) Intracellular cytokine staining after 8 days of PBMC culture with a pool of 36 HIV-1 peptides. HIV-specific CD4+ (upper panel) and CD8+ (lower panel) T cells able to produce IFNγ, TNFα and/or MIP1β. Plots are gated on viable CD3+ CD4+ or CD4+CD8+ T cells, respectively.

Research into a cure for HIV currently aims to remove any trace of HIV DNA from human cells, including through 'purging' of the latent HIV reservoir [3,13]. In contrast, we believe that the persistence of HIV DNA can lead to cure, and protection, from HIV. Globally, protection acquired by populations against viral infections was very commonly through integration of the infecting virus sequences and endogenization, as now also clearly described for bacteria and archaea with the CRISPR-Cas systems [14]. Retrovirus endogenization corresponds to retroviral DNA integration into germ cells, which allows its vertical transmission [15]. Endogenous retroviruses (ERV) are present in genomes from many mammals, including in humans; ≈8% of the genetic sequences of modern man are retroviral sequences, corresponding to ≈100 000 ERV [16,17]. The most famous ERV genes encode syncytins, which allowed syncytiotrophoblast formation in mammalian placentas [18]. Recently, retrovirus endogenization was highlighted as on-going in koala populations [19,20]. These findings suggest that without therapeutic and prophylactic strategies, after several decades of HIV/host interactions and millions of deaths, it is likely that a few individuals might have endogenized and neutralized the virus and transmitted it to their progeny. In fact, given our difficulties in obtaining HIV sequences from the two case-patients, we speculate that this phenomenon may be much more common than observed in sequence databases, and that HIV cure may have already occurred through endogenization, in default of occurring through HIV 'reservoir' eradication [3]. Massive sequencing in the near future of human DNA, including from African native individuals more extensively and anciently exposed to HIV, may reveal traces of such HIV endogenization processes. Duration of endogenization at the individual level is unknown.

Several mechanisms of resistance to HIV have been described [6,21]. Those involved in resistance to HIV super infection observed in case-patient 1 may include resistance conferred by integrated HIV DNA. Thus, resistance to exogenous retroviruses conferred by related ERV has been reported, which can include blocking of entry receptors or cell budding [22,23]. Regarding differences between anti-HIV immune responses observed on Western blotting for the two case-patients' sera, they may be related to different mechanisms of the endogenization process. Reactivities are weaker and fewer for case-patient 2, but this patient exhibits strong CD4+ T-cell responses. In addition, some viral

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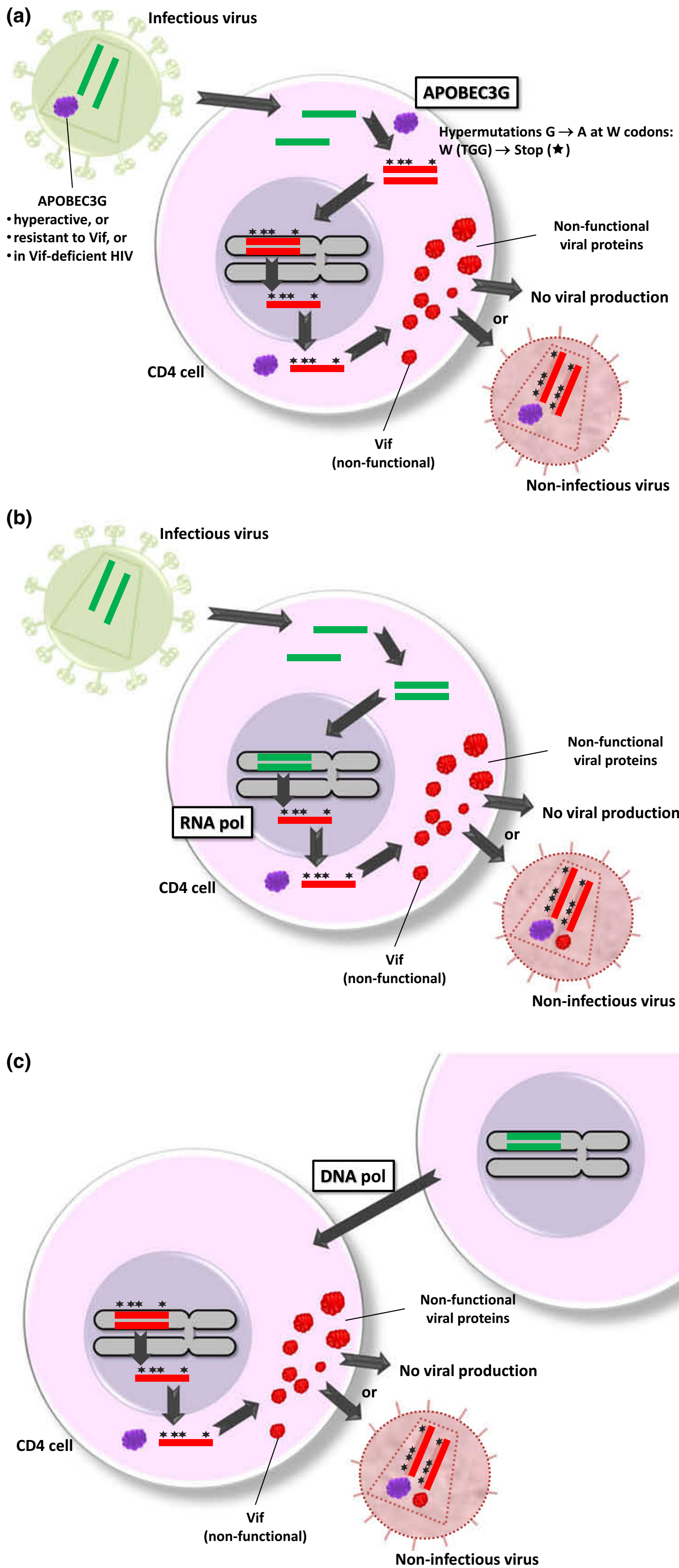

FIG. 4. Schematic of hypotheses for tryptophan (W)-to-stop codon mutations, and HIV cure, in the two case-patients. W-to-stop codon mutations may occur due to increased APOBEC3G activity (including that mediated, or boosted, by *vif* gene knockout) (a), errors of the cellular RNA polymerase (b) or DNA polymerase (c).

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proteins are still produced and may play a role in the maintenance of T-cell immunity detected after cell stimulation *in vitro* [24].

In our case-patients, we suspect that knock out (KO) of the viral genes by replacing a W codon by a stop codon is the key to understanding the viral neutralization. Indeed, between 16 and 24 W-to-stop mutations, including at positions only observed in ECs, were observed in HIV sequences [11]. The reason for these KO mutations is not established (Fig. 4). It may be that the patients' DNA or RNA polymerases are prone to errors that are not fatal for the patient's health. In any case, these patients had normal W levels in their blood. The best candidates are APOBEC proteins, and specifically APOBEC3G, which enters the virion and changes G to A [25], as observed here, and is thought to represent an ancient strategy of defence against ERV in humans [26]. These proteins may be more expressed or active in the two case-patients, which was not demonstrated here. Otherwise, APOBEC3G activity may have been temporarily boosted by interferon-alpha administered to the index case for his chronic hepatitis C [24,27]. In addition, a recent study suggests that co-infection with *Streptococcus* may enhance the activity of these proteins and inhibit HIV growth by generating hypermutability [28]. The Vif protein theoretically blocks APOBEC3G activity [29]. As the Vif encoding gene is KO at a functionally critical position (21) [30] in case-patient 1, this is likely to have contributed to the extensive inactivation of the HIV genome.

The phenomenon of HIV gene inactivation that precludes the production of replication-competent viruses has been previously reported by several teams, including recently by Eyzaguirre *et al*. [11], who stated that some of their patients had defective proviral genomes. What we searched for and found here were HIV-infected patients with integrated viral DNA in their genomes but no HIV production. We do not believe that our two case-patients are unique, or the phenomenon described here is new. In contrast, our approach largely differs from earlier ones, as we suggest that persistence of integrated HIV DNA is not a barrier, but on the contrary, may be a prerequisite for HIV cure. Therefore, we propose a new vision of HIV cure through integration, inactivation and potential endogenization of a viral genome into the human genome. Finally, we believe that potential mechanisms of the natural cure of or resistance to HIV through persistence of integrated defective HIV DNA and endogenization are a critical model to develop preventive and curative strategies. We suggest that testing the occurrence of stop codons in the DNA of the integrated HIV (including the two signatures in the vpr and env genes) may predict the control and/or cure of the disease. This is critical because, for all patients being treated, the natural resistance will no longer be apparent. In conclusion, our findings, which warrant further confirmation, are a first step in understanding the resistance to retroviruses. They may allow us to figure out the endogenization of retroviruses and detect resistant patients, as well as to initiate strategies that imitate these patients in order to cure or prevent AIDS.

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Transparency Declaration

The authors have no conflicts of interest to declare. The study was funded internally.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Supplementary results.

Figure S1. Phylogenetic tree based on HIV *pol* gene.

Figure S2. HIV-1 RNA detection post-stimulation of peripheral blood mononuclear cells from case-patient no. 2 by PHA and antibodies to CD3/CD28, IL2 and IL7.

Figure S3. Seroneutralization, by the serum of case-patients (a) and controls (b), of infection by the HIV-1 strain NL4.3 of peripheral blood mononuclear cells from HIV-negative donors.

Figure S4. Infectability of peripheral blood mononuclear cells from case-patient no.2 by the HIV-1 strain NL4.3.

Table S1. Distribution of tryptophan (W)-to-stop mutations in HIV genomes from the two case-patients, natural viral suppressors, HIV-infected controls on highly active antiretroviral treatment and HIV-infected untreated controls.

Table S2. Primers used for Sanger sequencing of HIV DNA from the two case-patients.

Table S3. Primers used for Sanger sequencing of patients' messenger RNA encoding prostaglandin E2 synthase, RNA polymerase and DNA polymerase.

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