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Electromagnetic Detection of HIV DNA in the Blood of AIDS Patients Treated by Antiretroviral Therapy

Luc MONTAGNIER[1,2], Jamal AÏSSA[2*], Claude LAVALLÉE[1],
Mireille MBAMY[3], Joseph VARON[4], Henri CHENAL[3]
[1](World Foundation for AIDS research and Prevention (UNESCO), 1 rue Miollis, 75015, Paris, France)
[2](Nanectis Biotechnologies, 98, rue Albert Calmette, 78350 Jouy-en-Josas, France)
[3](CIRBA Centre Intégré de Recherches Biocliniques d'Abidjan, Bd de Marseille, Km 4, BP 2071, 18 Abidjan, Ivory Coast)
[4](The University of Texas Health Science Center, 2219 Dorrington Street, Houston, Texas 77030, USA)

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Abstract: Electromagnetic signals of low frequency have been shown to be durably produced in aqueous dilutions of the Human Imunodeficiency Virus DNA. In vivo, HIV DNA signals are detected only in patients previously treated by antiretroviral therapy and having no detectable viral RNA copies in their blood. We suggest that the treatment of AIDS patients pushes the virus towards a new mode of replication implying only DNA, thus forming a reservoir insensitive to retroviral inhibitors. Implications for new approaches aimed at eradicating HIV infection are discussed.
Key words: HIV DNA, antiretroviral therapy, HIV reservoir, HIV LTR detection, HIV DNA with erythrocyte fraction.

1 Introduction

Antiretroviral therapy (ART) is now the standard treatment of HIV infected patients (Zuniga *et al.*, 2008). Generally composed of three or four inhibitors of the viral reverse transcriptase and protease, it results in a quasi complete disappearance of the HIV viremia, or measured by the strong reduction of viral RNA copies (viral load) in the patient's serum.

The limit of detection of RNA copies by commercial kits (200 or 40) is usually attained within 3 to 6 months when the virus is fully sensitive to the viral inhibitors.

However, as soon as the treatment is interrupted, virus multiplication resumes within weeks, as evidenced by the increase of the virus load and the decrease of the CD4 T cell numbers.

This indicates that there is a viral reservoir to which the inhibitors have no access or no effect. This reservoir is presumably made of proviral DNA integrated in cells in a dormant state (Douglas *et al.*, 2009).

In this study we show that ART treatment of patients induces the release into their blood of HIV DNA sequences detectable by a new biophysical technology that we have previously described for bacterial DNA (Montagnier *et al.*, 2009).

Our data suggests that inhibition of infection at the reverse transcription step is pushing the virus towards a low level of replication using only DNA templates.

This would explain why the classical inhibitors used in ART cannot achieve eradication of the viral infection.

We have previously reported (Montagnier *et al.*, 2009) the detection of electromagnetic waves of low frequency by high dilutions in water of the DNA of pathogenic bacteria. This is a resonance phenomenon likely to be produced by polymerized water molecules organized by some DNA sequences. We wondered whether the genetic material of viruses could induce the same transformation of water, particularly that of HIV.

We set up in vitro experiments in which CEM cells were infected with our prototype HIV1 strain, HIV LAI. Prior to the experiments, cells and infecting virus were first checked for mycoplasma contamination by using a highly sensitive polymerase chain reaction (PCR) technology based on 16 s ribosomal RNA (Montagnier and Lavallée, 2007). Traces of Mycoplasma arginini were found only in control CEM cells, but no electromagnetic signals (EMS) could be detected in the culture supernatant of such cells.

By contrast, EMS were detected in dilutions of the culture supernatant of the HIV-infected cells, when the cytopathic effect was obvious. Filtration through 20 nM filters was found to be necessary to detect the EMS, indicating that the source of signals was smaller than this size and therefore smaller than the intact virus par-

*Corresponding author.
E-mail: jaissa@netcourrier.com

ticles whose diameter ranges between 100 to 120 nM.

In order to evaluate the density of such particles, an aliquot of the infected CEM supernatant was centrifuged to equilibrium on a sucrose density gradient, in conditions where HIV virions form a sharp band at the density of 1.16.

By contrast, the nanoparticles producing the EMS were associated with fractions ranging in densities from 1.15 to 1.25.

A longer time of centrifugation used to improve the density equilibrium did not modify this profile.

2 EMS in the plasma of infected and treated patients:

Plasmas were prepared from heparinized blood of patients presenting with different conditions:

-Asymptomatic, untreated;

-Symptomatic, not yet treated, with high virus load;

-Symptomatic, treated by antiretroviral therapies with no detectable virus load by commercial kits (<200 RNA copies/ml). A total of 125 patients were studied.

We regularly detected EMS (higher frequency than background) only in the plasma of the third category (30 out of 30), in plasma dilutions ranging from 10^{-4} to 10^{-8} (Fig. 1). Results with the two first categories were generally negative, with the exception of one untreated AIDS patient.

We determined the conditions of preparation and storage of the plasma samples for optimizing the capture of EMS. The plasma had to be kept unfrozen, preferentially stored at +4 °C. Freezing and storing at −20 °C or −80 °C destroyed their capacity to produce EMS, unless we had extracted the primary source of the signals, DNA (see below).

Serums taken from the clotted blood were also negative, whether kept at +4 °C or frozen.

Heating of the diluted 10^{-2} plasma dilutions at 65 °C for one hour also inactivated or reduced significantly their ability to produce EMS.

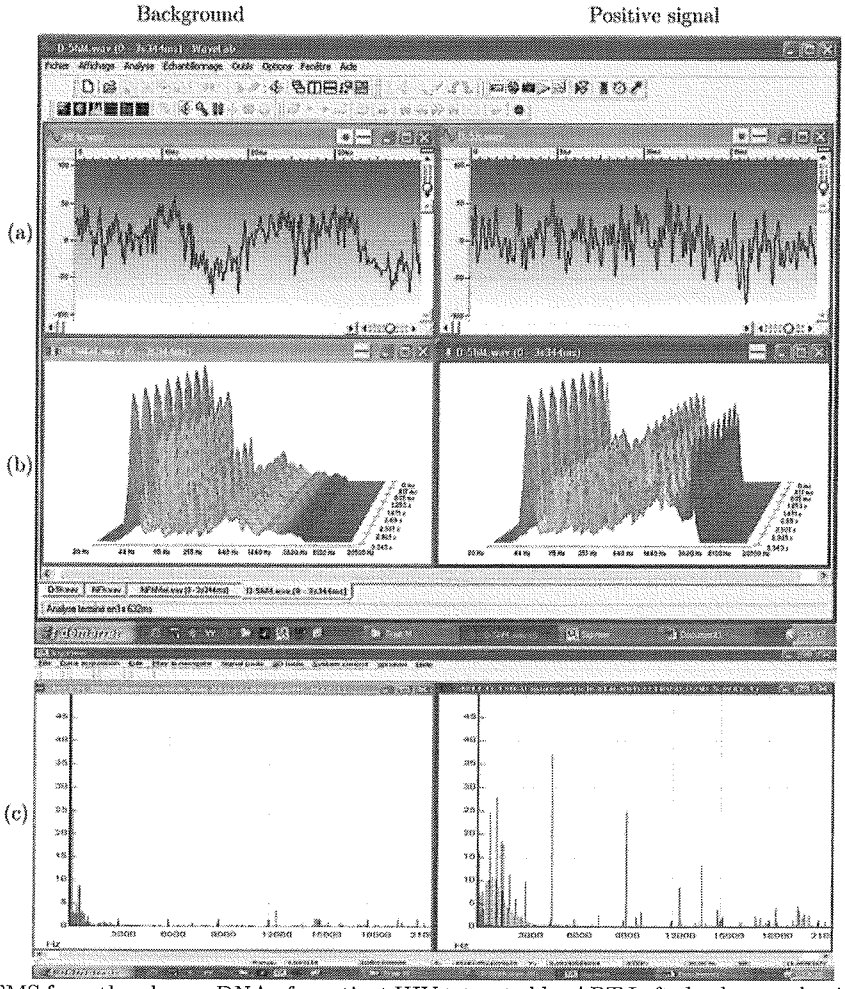


Fig. 1 Detection of EMS from the plasma DNA of a patient HIV+ treated by ART Left: background noise (from an unfiltered suspension or a negative low dilution) Right: positive signal [from a 10^{-5} (D-5)] dilution (a) detailed analyzis of the signal (scale in millisecondes); (b) Matlab 3D Fourier transform analysis (abcissa: 0-20 KHz, ordinate: relative intensity, 3D dimension: recording at different times Frequencies are visualized in different colors; (c) Sigview Fourier transform: note the new harmonics in the range of 1 000-3 000 Hz

We studied the decay with time of EMS production by the plasma stored at +4 °C. As shown in Fig. 2, the emission capacity could last for several days, sometimes for several weeks of storage, indicating a relative stability of the nanostructures in the plasma proteinic environment.

As for the in vitro studies, filtration of the plasma (usually at the 1/100 dilution in PBS or saline) through 20 nM filters was a prerequisite for detecting the signals in further dilutions of water.

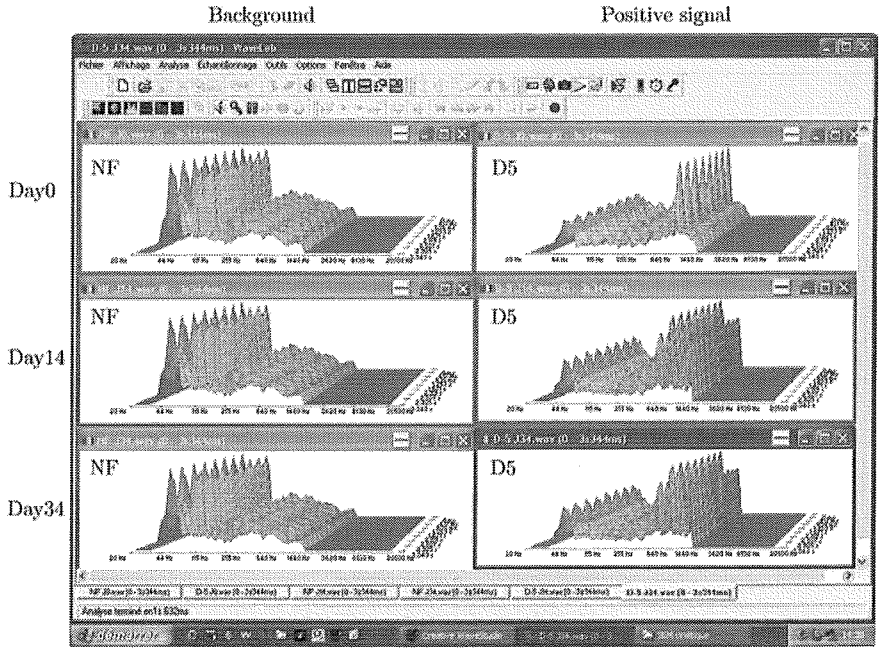


Fig. 2 Relative stability of EMS emission by the plasma of a patient HIV+ treated by ART according to the time of storage at 4 °C (Matlab analysis conditions as Fig. 1) Left: NF: Unfiltered suspension Right: positive signal [from a 10^{-5} (D5)] dilution

In some rare cases, we could detect weaker signals at lower dilutions after filtration through 100 nM porosity filters.

3 HIV DNA is the source of EMS:

We had previously identified some DNA sequences as the source of bacterial EMS (Montagnier *et al.*, 2009). We therefore asked whether nucleic acids carrying the genetic information for HIV, either residual viral RNA or proviral DNA, could be the sources of signals in the plasma of infected patients.

We again choose to study three different groups of patients infected and not treated in the asymptomatic or symptomatic stages, or infected and treated with ART with no detectable viral load. Nucleic acids (NA) were extracted from plasmas by the phenol-chloroform method from plasmas diluted 1/100 in PBS. Ethanol precipitates were solubilized in water and the solutions were filtered through 20 nM filters.

DNA concentrations were adjusted to be 1 to 4ng/ml of water-Tris-HCl 10 mM.

EMS were detected only in the group of patients treated by antiretroviral therapy and having undetectable virus load. The signals were produced in the same range of aqueous dilutions than fresh plasma (Table 1 and Fig. 3). Filtration of the original solution (1/100 dilution) and vortex agitation of each of the further aqueous dilutions were necessary in order to capture the EMS emission.

Treatment by RNase (10 µg/ml, 1 h at 37 °C) of the original solution had no effect, suggesting that DNA rather than viral RNA was involved in EMS production. This was confirmed by the inactivating effect of DNase, provided the nanostructures previously induced by DNA in water before DNase treatment was abolished by freezing. On the contrary, DNA molecules are not affected by freezing and can reinduce the water nanostructures after freezing.

The experimental protocol and results are shown in the following chart:

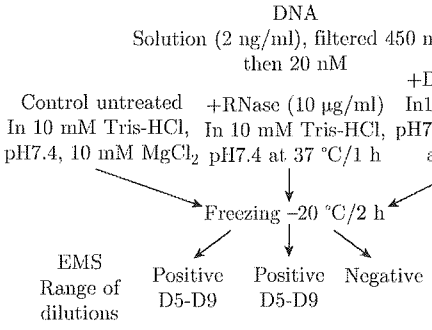


Table 1 **Detection of EMS from HIV DNA in infected patients with different conditions. B1, B2, B3, B4: Asymptomatic untreated; C1, C2, C3, C4: Symptomatic treated by ART; D1, D2, D3, D4: Symptomatic untreated; ND: Not done; RBC: Red Blood Cells; VVBC: White Blood Cells; 3TC: Epivir® (Lamiduvine); FTC: Emtrive® (Emtricitabine); TDF: Viréal® (Tenofovir); AZT: Rétrovir® (Zidovudine); EFV: Sustiva® (Efavirenz); EMS: Electromagnetic signals**

Patient	Age	Clinical category	Treatment ARV	CV (copies/ml)	CD4/mm3		EMS	Positive dilutions (D)
B1	40	Asymptomatic untreated		180 000	416	plasma	-	
						plasma DNA	-	
						WBC DNA	-	
						RBC DNA	-	
B2	57	Asymptomatic untreated		ND	338	plasma	-	
						plasma DNA	-	
						WBC DNA	-	
						RBC DNA	-	
B3	22	Asymptomatic untreated		ND	509	plasma DNA	-	
B4	58	Asymptomatic untreated		ND	221	plasma DNA	-	
C1	33	Symptomatic treated	TDF+FTC+EFV	< 200	520	plasma	+	D-4 to D-7
						plasma DNA	+	D-4 to D-8
						WBC DNA	-	
						RBC DNA	+	D-5 to D-9
C2	45	Symptomatic treated	AZT+3TC+EFV	< 200	381	plasma	+	D-5 to D-9
						plasma DNA	+	D-5 to D-9
						WBC DNA	-	
						RBC DNA	+	D-5 to D-8
C3	44	Symptomatic treated	AZT+3TC+EFV	< 200	289	plasma	+	D-6 to D-9
						plasma DNA	+	D-3 to D-7
						WBC DNA	-	
						RBC DNA	+	D-4 to D-8
C4	51	Symptomatic treated	AZT+3TC+EFV	< 40	258	plasma	ND	
						plasma DNA	+	D-5 to D-9
						WBC DNA	ND	
						RBC DNA	+	D-5 to D-8
D1	39	Symptomatic, not yet treated		ND	93	plasma	-	
						plasma DNA	-	
						WBC DNA	-	
						RBC DNA	-	
D2	32	Symptomatic, not yet treated		ND	162	plasma DNA	-	
D3	27	Symptomatic, not yet treated		ND	153	plasma DNA	-	
D4	55	Symptomatic, not yet treated		ND	27	plasma DNA	-	

3.1 Location of the active DNA in blood fractions

The heparinized blood of several HIV+ ART-treated patients was run on a Ficoll gradient. DNA was extracted from the three main fractions: plasma (with platelets), white cells layer and the erythrocyte pellet. Each DNA extract was tested for EMS emission.

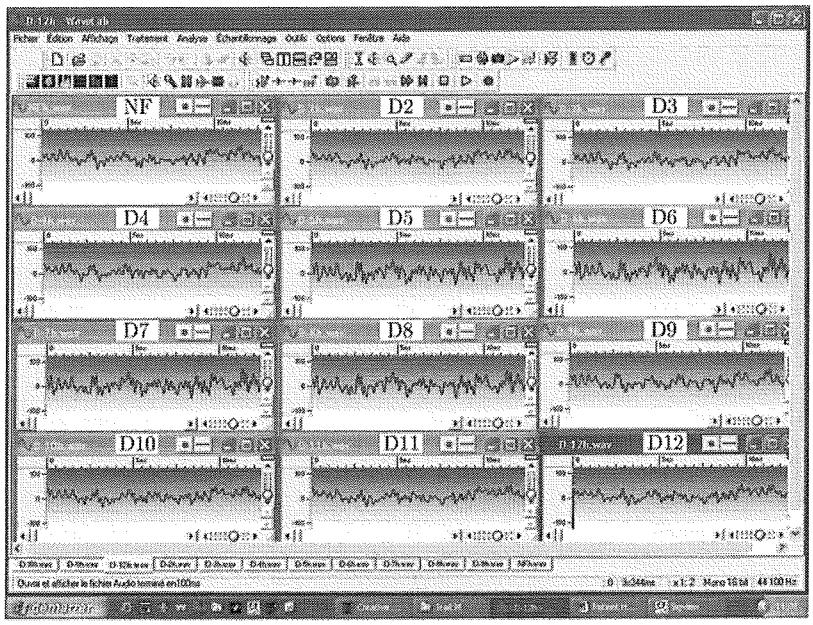
In all the patients with undetectable virus load, only

the DNA from the plasma and the erythrocyte fractions gave strongly positive signals. The white cell layer-derived DNA gave no or weak signals. In ART-treated patients with remaining high virus load, only the plasma-derived DNA was positive (not shown).

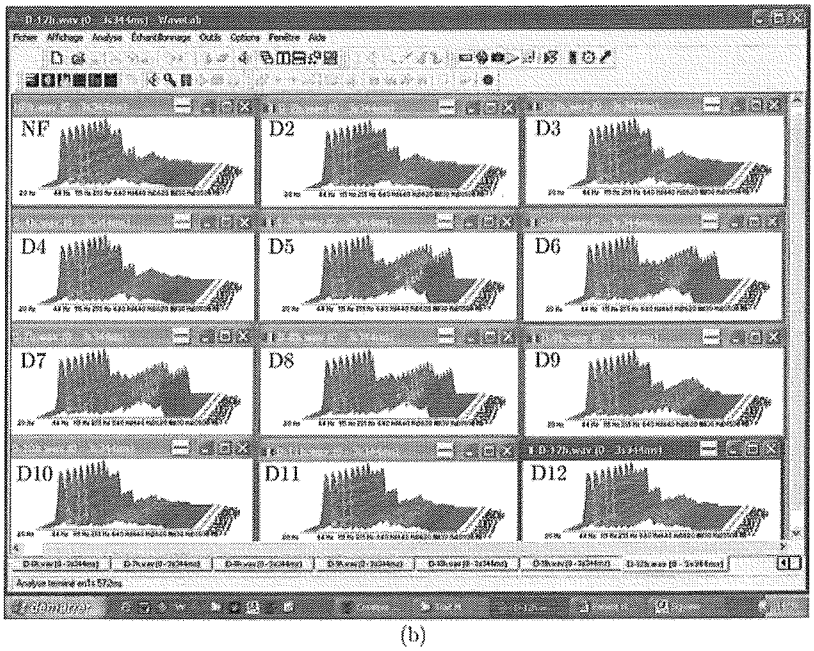
4 Nature of the HIV sequences at the origin of EMS

We learned from our previous work on the adhesin gene of Mycoplasma pirum (Montagnier *et al.*, 2009) that a single gene or even a fragment of a gene is sufficient to produce the EMS. We therefore used an infectious DNA clone of HIV, previously constructed in our laboratory from HIV Lai (Peden *et al.*, 1991) to determine which part of the viral genome was at the origin of EMS. To this end, we designed some specific primers for sorting out by PCR the main sequences corresponding to the different structural and regulatory genes of HIV, including LTR, POL, GAG, ENV, NEF and VIF.

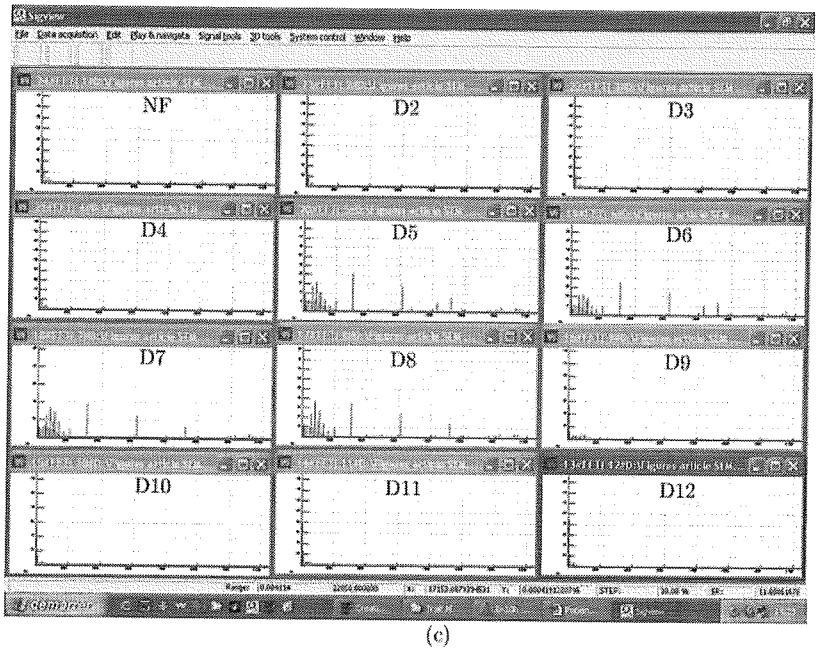
The amplicons and secondary amplicons resulting from nested-PCR were analyzed by agarose gel electrophoresis and yielded the expected fragment sizes. The DNA bands were extracted and purified, and assayed for EMS production at different dilutions. As a control, the entire proviral HIV DNA genome was also



(a)



(b)



(c)

Fig. 3 A typical recording of signals from aqueous dilutions of the plasma DNA of a patient HIV+ treated by ART (Matlab software): note the positive signals from D^{-5} to D^{-8} dilutions: (a) millisecond analysis; (b) Fourier transform analysis Matlab; (c) Fourier transform analysis SigView

tested and found positive for EMS. Table 2 summarized the results, indicating that several sequences are the source of EMS: LTR, NEF and ENV.

The same primers were used to detect specific sequences in the DNA extracted from the plasma or the red blood cell pellet of the positive patients. The amplified LTR DNA fragment, visualized as a band of 104 bp by nested PCR, was constantly found in all preparations, followed unfrequently by NEF and ENV amplified fragments (Table 2). Sequencing of the LTR band confirmed its HIV origin with 99% identity with the prototype HIV DNA (2 nucleotide differences out of 104). Interestingly, a higher sensitivity of detection was obtained by the use of reverse transcriptase (RT) before the use of the Taq polymerase in the PCR reaction.

However this reaction was not affected by prior RNAse treatment, indicating that a DNA template not RNA, was also used by the RT enzyme.

In addition when aqueous dilutions were tested, an x10-fold to 100-fold increase (1 to 2 decimal dilutions) of sensitivity was obtained, when each dilution was strongly agitated by vortex, as for the detection of EMS.

5 Discussion

We have adapted the technology previously described for the capture of electromagnetic signals induced by bacterial DNA to the detection of HIV DNA sequences. Unlike the nanostructures induced in water by bacterial DNA which passed through 100 nM filters, but were retained by 20 nM filters, the HIV DNA nanostructures are smaller as they passed through 20 nM filters. The range of dilutions at which the EMS could be detected are lower, starting from 10^{-3} up to 10^{-9} decimal dilutions. There is no detectable difference in the profile of the signals at this level of our technology, indicating that we are dealing again with a resonance phenomenon of water polymers.

The material structures at the origin of the signals are unlikely to come from mature HIV virions, as they differ in density in sucrose gradient from the density of 1.16 of retroviruses. Moreover, in the blood of AIDS patients, they are produced by DNA and not RNA.

The most important result of this paper, apparently paradoxical, is that only HIV-related DNA sequences from patients treated with antiretroviral therapy and having no detectable RNA in their blood can be detected by EMS emission and by PCR. Naive untreated patients show no evidence of such DNA. This result was obtained with patients of different geographic locations (North America, Europe, West and Central Africa) presumably infected with different HIV subtypes.

Interestingly, this DNA is not only detected in the plasma fraction, but also found associated with the erythrocyte fraction. As there is no DNA in mature erythrocytes, the viral DNA is probably present in nanostructures bound to the erythrocyte membrane (exosomes) or in nucleated cells that sedimented with the erythrocytes (granulocytes?). In treated patients having still a detectable virus load, the DNA was only found in the plasma fraction.

Table 2 Detection of EMS from amplified HIV DNA obtained by PCR and n-PCR. ND: not done; PCR: Polymerase Chain Reaction; n-PCR: nested-PCR; EMS: Electromagnetic signals

HIV GENES		Sequence range	N° of base pair (bp)	DNA	EMS	Positive dilutions (D)
GAG	PCR	890→2 278	1 388	Clone	-	
				Patient (C1)	-	
	n-PCR	1 491→2 038	547	Clone	-	
				Patient (C1)	-	
POL	PCR	3 219→4 805	1 586	Clone	-	
				Patient (C1)	-	
	n-PCR	4 174→4 669	495	Clone	-	
				Patient (C1)	-	
ENV	PCR	6 438→7 816	1 378	Clone	-	
				Patient (C1)	-	
	n-PCR	6 848→7 521	673	Clone	+	D-4 to D-8
				Patient (C1)	-	
LTR	PCR	83→570	487	Clone	-	
				Patient (C1)	-	
	n-PCR	445→549	104	Clone	+	D-4 to D-8
				Patient (C1)	+	D-4 to D-8
NEF	PCR	8 797→9 398	601	Clone	-	
				Patient (C1)	-	
	n-PCR	9 066→9 280	214	Clone	+	D-5 to D-9
				Patient	+	D-4 to D-7

What could be the source(s) of this DNA and its possible role?

PCR analysis of the prototype HIV1 Lai DNA indicates that short fragments of nested-PCR amplified DNA, in picogram amounts, are the source of EMS, derived in particular from the LTR, POL and ENV genes. Similarly, in the case of patient DNA, the LTR and NEF-derived amplicons were also EMS positive.

There may be in vivo DNA fragments corresponding to other genes which are not picked-up by the primers that we have been using. Therefore it is possible that the whole genome is represented as DNA fragments in the blood or even as an entire genomic molecule. The most simplistic explanation for the presence of this DNA is that it reflects the breakdown (apoptotic?) of some infected cells containing the proviral DNA in a latent state. This would imply that after antiretroviral treatment, these cells die and constitute a reservoir large enough to be continuously refilled by new living cells. A priori, there is no reason that such cells, unless they express some HIV proteins recognized by cytotoxic T cells, will be destroyed by the immune reactions.

In another hypothesis, the DNA detected represents forms of unintegrated HIV DNA. Various circular DNA forms have been described during HIV infection in vitro and in vivo (Meyerhans *et al.*, 2003). Sharkey *et al.* (2000) have even described the persistence of episomal forms of HIV DNA in some patients treated by antiretroviral therapy with undetectable viral RNA in their blood.

However their study was focalized in peripheral blood mononuclear cells (PBMC)

By contrast, we could not detect our HIV DNA in PBMC, indicating it comes from other cell types and tissues.

We therefore will favor a third hypothesis:

The antiretroviral therapy works efficiently to prevent reverse transcription of viral RNA into DNA and therefore blocks any productive infection of susceptible cells. However it will not prevent DNA-DNA replication in a non-integrated state. In other words, we hypothesize that the ART treatment will push the virus towards an alternate way of replication, probably minor and depending on a cellular DNA polymerase, but sufficient to maintain the viral genetic information as unintegrated viral DNA and able to resume the normal viral cycle if ART is interrupted for any reason.

The DNA found in the blood circulation would then be a by-product of this DNA. The cells and tissues in which this DNA replication occurs remain to be identified.

This hypothesis, if confirmed, would have some important implication for the eradication of HIV infection. If we can target by specific inhibitors this episomal replication, without damaging the cellular processes, we might achieve complete elimination of

the HIV reservoir and therefore eradication of HIV infection.

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Appendix

Experimental Section

1 Fractionation on Ficoll gradient

Peripheral whole blood from patients who had given their informed consent, was collected in Vacutainer tubes containing lithium heparin, 3 ml of whole blood were diluted with 10 ml phosphate buffered saline (PBS) buffer and layered over 3ml of Ficoll-paque (1.077 g/ml density; Amersharm Biosciences) in 15 ml leucosep® tubes and centrifuged at 1000 x g for 10 min at 4 °C. Plasma was removed, the red blood cell (RBC) pellet and the white blood cells (WBC) were washed 2 times with 10 ml of PBS and centrifuged at 250 x g.

2 DNA extraction

Plasma DNA, WBC DNA and RBC DNA were extracted by Proteinase K in the presence of SDS (sodium dodecyl sulfate) and further deproteinized by phenol-chloroform mixture. The pellet obtained by ethanol precipitation was resuspended in Tris 10-2 M, pH 7.6 and an aliquot was diluted 1/100 in water. The dilution (10-2) was filtered first through a 450 nM filter and the resulting filtrate was then filtered again on a 20 nM filter Anotop (Whatman). The filtrate was further diluted in serial decimal dilutions in water as previously described (Montagnier *et al.*, 2009).

3 EMS measurement

For capture and analysis of EMS, a coil, bobbin of copper wire, was used and connected to a Sound Blaster Card itself connected to a laptop computer, preferentially powered by its 12 volt battery as previously described (Montagnier *et al.*, 2009). Each emission was recorded twice for 6 seconds, amplified 500 times and processed with different softwares for visualization of the signals on the computer's screen. The main harmonics of the complex signals were analyzed by utilizing several softwares of Fourier transformation.

4 PCR primers

PCR primer sequences were derived from HIV genomic sequence data bank (http://www.hiv.lanl.gov/) and synthesized by Invitrogen (France). UV absorbance was used to assess the quality of primer synthesis.

One-step reverse transcriptase (RT)-PCR experiments were performed with the mastercycler® ep (Eppendorf). A 50 µl RT reaction included 25 µl of 2xRT-PCR buffer, 16.6 µl of nuclease-free-water, 0.4 µl of 25 mM of each deoxynucleoside triphosphate (dNTPs), 1 µl of 50 µM of each appropriate primer (Invitrogen), 1-4 ng/ml of total DNA and 1 µl of iScript RT (BioRad). The RT-PCR mixtures were incubated at 42 °C for 30 min (RT step) followed by 1 cycle (inactivation and denaturation step) at 95 °C for 3 min, followed by 42 PCR cycles of amplification (95 °C for 30 s; 56 °C for 30 s; 78 °C for 2 min). A final extension step was performed at 78 °C for 10 min.

The nested-PCR mixture (50 µl) contained 29.4 µl of nuclease-water-free, 5 µl of 10x taq PCR buffer, 8 µl of 25 mM $MgCl_2$, 0.4 µl of 25 mM dNTPs, 1 µl of 50 µM each appropriate primer, 5 µl of RT-PCR product and 1 µl of 5 U/µl Taq DNA polymerase (Invitrogen).

The nested-PCR was performed with the mastercycler® ep (Eppendorf). The nested-PCR mixtures were pre-heated at 95 °C for 3 min (inactivation and denaturation step), followed by 42 PCR cycles of amplification (95 °C for 30 s; 56 °C for 30 s; 78 °C for 2 min). A final extension step was performed at 78 °C for 10 min.

The amplification products were separated on a 1.2-1.5% agarose gel electrophoresis / EtBr gel and visualized using a Molecular Imager® Gel DocTM XR System (BioRad).

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