Polymerase chain reaction amplified HTLV-I, HIV-1 and HIV-2 DNA fragments in subjects with mixed retroviral infections

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Summary. Serum and peripheral blood mononuclear cells from eight patients from the Ivory Coast with positive screening test results for retroviral infections were studied by serology (ELISA, Western blot (WB), synthetic peptide test), cell co-culture, and polymerase chain reaction (PCR). Two HIV-2 infections with indeterminate interpretation on HIV-1 WB were detected, two were clear dual HIV-1/HIV-2 infections, three were ambiguous mixed HIV-1/HIV-2 infections, and one was a triple retroviral infection by HTLV-I, HIV-1 and HIV-2. Four slow/low HIV-1 strains were isolated at the expense of HTLV-I and HIV-2 strains. The ELISA tests were found to be very sensitive. Indeterminate WB interpretations were frequent (HTLV-I, four; HIV-1, three; HIV-2, two). PCR provided clear evidence of multiple retroviral infections in three cases and enabled interpretation of indeterminate WB samples in three cases. One sample presented a puzzling pattern with positive PCR results for HIV-1 and HIV-2 associated with negative or indeterminate serological results. Thus, our data emphasise the need to analyse serological as well as virological markers to gain better insight on mixed retroviral infections, especially in endemic areas such as West Africa.

Introduction

The human T-cell leukaemia virus type I (HTLV-I) was the first human retrovirus discovered. HTLV-I, first isolated from a patient thought to have mycosis fungoides,¹ has been strongly associated with a rare form of lymphoma called adult T-cell leukaemia/lymphoma (ATL).² The human immunodeficiency viruses, type 1 (HIV-1) and type 2 (HIV-2), are causative agents of the acquired immunodeficiency syndrome (AIDS).^{3,4}

Geographic distributions of these three major human retroviruses are different. HTLV-I is endemic in Southwestern Japan, the Caribbean basin and sub-Saharan Africa.⁵ HIV-1 appears as a worldwide epidemic, whereas HIV-2 has been mainly reported in West Africa.⁶ Numerous seroprevalence surveys have reported mixed retroviral infections.^{7,8} However, serological techniques did not always allow clear discrimination between the multiple infections. These patterns are especially frequent in West Africa.⁸

In this report, data obtained with serology, cell co-culture and polymerase chain reaction (PCR) on samples from Ivory Coast subjects showing positive screening test results for co-infection are examined.

Materials and methods

Samples

Serum and peripheral blood mononuclear cells (PBMCs) were collected in 1988 from eight subjects (nos. 1–8) residing in Abidjan, Ivory Coast. None of these (four males and four females) was diagnosed as having AIDS or ATL. Three subjects presented with sickle-cell anaemia and one with an infiltration of the upper lip, stains on the sole of the foot and palm of the hand. The others were healthy individuals.

ELISA

Sera were screened by ELISA for antibodies to HTLV-I/II (HTLV-I Elisa; DuPont/Biotech, Wilmington, DE, USA), HIV-1 (HIV-1/HIV-2 EIA recombinant; Abbott, North Chicago, IL, USA) and HIV-2 (Elavia Ac-Ab-Ak II; Diagnostics Pasteur, Marnes-la-Coquette, France). The procedures and evaluations were done according to the manufacturers' instructions.

Western blot (WB)

All sera were examined by WB testing (DuPont/

Biotech and Cambridge Biotech for HTLV-I/II; DuPont/Biotech for HIV-1; Diagnostics Pasteur for HIV-2). The WB assays were performed according to the manufacturers' instructions.

In WB HTLV-I strips from Cambridge Biotech, native viral polypeptides were combined with a purified recombinant transmembrane envelope protein (p21) to increase the sensitivity for *env* antibodies and the specificity to encompass HTLV-II reactive antibodies.⁹ The criterion for a serum to be considered HTLV-I/II positive by WB analysis was the presence of gp61 or gp46 or p21 reactivity with, at least, p19 or p24 reactivity. Sera without any WB reactivity (i.e., blank) were considered to be negative. All other WB profiles were labelled as indeterminate.

Samples were considered to be positive for antibodies to HIV-1 or HIV-2 if they reacted with at least two envelope proteins and one of the *gag* proteins.

Synthetic peptide test

The Pepti-LAV 1-2 test (Diagnostics Pasteur) was used to discriminate serologically between HIV-1 and HIV-2 infection. This test is based on the use of two synthetic peptides.¹⁰ HIV-1 peptide imitates an immunodominant epitope of the gp41 envelope glycoprotein. HIV-2 peptide imitates an epitope of the gp36 envelope glycoprotein. The assay was performed according to the manufacturer's instructions. The following criteria were used for reading band intensities: the control band (IgG control) corresponded to a response intensity arbitrarily labelled as (+ +); each band was scored by eye by comparison with the intensity obtained on the control band; any positive band with an intensity equal to or greater than or lower than that of the control band was labelled as (+ +) or (+), respectively; any minor distinct staining was marked as (+/-). Absence of reactivity corresponded to a negative response.

Cell co-culture

Peripheral blood mononuclear cells (PBMCs) from patients and healthy seronegative subjects were collected on a Ficoll gradient from 30-ml samples of heparinised blood. PBMCs from healthy donors were stimulated by exposure to phytohaemagglutinin (PHA; Difco) 0.1% for 3 days in RPMI 1640 medium containing fetal calf serum 10%, penicillin 100 U/ml and streptomycin 100 µg/ml. Then, 5×10^6 PHA-stimulated PBMCs were added to 10^7 PBMCs from a patient and cultured in RPMI 1640 medium with fetal calf serum 10%, antibiotics and interleukin 2 (Transgene, USA) 250 U/ml. Tissue-culture flasks were incubated at 37°C in air with CO₂ 5%. At twice weekly intervals, half of the cells were split, enumerated with Trypan blue, and checked for reverse transcriptase (RT) activity. An equal volume of medium was added to the rest of the cells to continue the culture and a

further inoculum of PHA-stimulated PBMCs was added once a week.

HTLV-I, HIV-1 and HIV-2 antigens were detected by indirect immunofluorescence assay with serum from infected patients.

DNA preparation

The PBMCs obtained by Ficoll separation were washed twice in phosphate-buffered saline (PBS). The final pellet was suspended in lysis buffer containing 10 mM Tris-HCl, pH 8.0; 10 mM EDTA, pH 8.0; 10 mM NaCl, SDS 0.5% and proteinase K (Boehringer, Mannheim, Germany) 250 µg/ml and incubated overnight at 37°C. After phenol-chloroform extraction, the DNA was precipitated with 0.1 volume of 3 M sodium acetate and 2 volumes of absolute ethanol at -80°C for at least 1 h. The DNA was pelleted by centrifugation at 2000 g for 30 min, washed twice with ethanol 70% and dried under vacuum.

PCR

The PCR was performed as described previously.¹¹ Two pairs of oligonucleotides in the *gag* and *pol* regions of the HIV-1 genome, two pairs in the *pol* and *env* regions of the HIV-2 genome, and one pair in the *gag* region of the HTLV-I genome were selected as primers. The characteristics of the primers used (The Midland Certified Reagent Company, USA) are shown in table I.

The PCR mixture (100 µl) contained PBMC DNA 1 µg, 1 µM each of primers, 200 µM each of four dNTPs, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, gelatin 0.001% w/v, and AmpliTaq polymerase (Perkin Elmer Cetus, Norwalk, CT, USA) 2.5 units. The amplification was performed with a DNA thermal cycler (Perkin Elmer Cetus). The PCR procedure involved an initial denaturation step at 94°C for 10 min followed by 25 cycles of 2 min at 53°C for annealing, 3 min at 72°C for extension, and 1 min at 94°C for denaturation, and a final cycle with the extension time increased to 10 min.

Analysis of PCR products

The PCR product (10 µl) was resolved by electrophoresis on agarose 1.8% gel. After denaturing and neutralising steps, the gel was blotted on to a nitrocellulose membrane (Schleicher and Schuell, Keene, NH, USA). Hybridisation was performed with a ³²P-labelled probe and an oligolabelling kit (Pharmacia, Uppsala, Sweden). The HTLV-I probe was an *Sst*I-*Sst*I fragment from the *pMT-2* clone (Biotech); the HIV-1 probe was an *Sst*I-*Sst*I fragment from the *BH-10* clone;¹³ and the HIV-2 probe was an *Stu*I-*Stu*I fragment from the HIV-2 NIH-DZ clone.¹⁴ Hybridisation was performed in the presence of 2 × SSC, dextran sulphate 10%, SDS 1%, Denhart solution

Table I. Primers for DNA amplification by PCR

Primer designation		Sequence (5'-3')		Location*
HTLV-I	1	CGACCGCCCCGGGGGCTGGCCGCT	<i>gag</i>	863-886
	2	TATCCTTTTGGGAGTAGGCTGGC	<i>gag</i>	1050-1028
HIV-1	3	ATAATCCACCTATCCCAGTAGGAGAAAT	<i>gag</i>	1543-1570
	4	TTTGGTCCTTGTCTTATGTCCAGAATGC	<i>gag</i>	1657-1630
	5	TGGGAAGTTCAATTAGGAATACCAC	<i>pol</i>	2810-2834
	6	CCTACATACAAATCATCCATGTATT	<i>pol</i>	3117-3093
HIV-2	7	CCATACCACTACATGAGGAC	<i>pol</i>	2722-2741
	8	GCAACTTCCATTTAGTTGGC	<i>pol</i>	3096-3077
	9	TTGGCTTTAATGGCACTA	<i>env</i>	6914-6931
	10	AGGAACCAAGTCATGTTGCA	<i>env</i>	7322-7303

*The locations were determined from the sequence published by Myers *et al.*¹²

Fig. 1. **a**, HTLV-I WB patterns obtained with DuPont/Biotech assay for sera nos. 1-8. **b**, HTLV-I WB pattern obtained with Cambridge Biotech assay for serum no. 5; this test includes the recombinant p21 *env* protein.

5%, 20 mM Na_2HPO_4 , pH 6.5, and salmon sperm DNA 100 $\mu\text{g}/\text{ml}$ at 45°C overnight. Then, the filters were washed in $2 \times \text{SSC}$, SDS 0.1% for 2 h at 45°C, and exposed to Kodak XAR film overnight. The film was developed and the filters were washed a second time if necessary at 55°C with the same buffer and re-exposed to Kodak film.

Results

Immunoserology

HIV ELISA screening tests showed very strong positive results ($\text{OD} > 1.000$ with a cut-off at 0.180 for HIV-1 and 0.300 for HIV-2) for all sera, except no. 4

Fig. 2. HIV-1 WB patterns obtained for sera nos. 1-8.

for HIV-1 and no. 7 for HIV-2. Only serum no. 5 was positive in the HTLV-I ELISA (OD 1.800 with a cut-off at 0.741).

The result with serum no. 5 was confirmed by strong reactions in many bands in the HTLV-I WB (fig. 1). However, five samples showed indeterminate WB patterns: no. 1 with a p24 band alone; no. 4 with p32 and p53 bands and another reactivity located higher than the p53 band; nos. 6 and 8 with the p26 band alone. Serum no. 5 was reblotted with a strip that included the recombinant p21 *env* protein; the intensity of the p24 band was greater than that of the p19 band.

Sera nos. 1, 3, 5, 6 and 8 had complete HIV-1 WB profiles except that p15 reactivity was missing with nos. 1 and 3 (fig. 2). Three samples showed indeterminate WB profiles. Among these, an unusual pattern was observed with sample no. 4, and samples nos. 2 and 7 had single bands only at p24 and p31, respectively.

Sera nos. 1-5 presented HIV-2 patterns compatible with an HIV-2 infection (fig. 3). All these samples had a characteristic gp105 reactivity corresponding to an antigen encoded by the *env* gene. Intensity was weaker with sera nos. 3 and 4. Indeterminate patterns were observed with serum no. 6 (p26, p56 and p68 bands)

Fig. 3. HIV-2 WB patterns obtained for sera nos. 1-8.

Fig. 4. Results of the synthetic peptide test for HIV-1/HIV-2 antibody differentiation for samples nos. 1, 3, 4, 5, 6 and 7.

and no. 8 (p26 band alone). Sample no. 7 gave a completely blank result.

The results obtained with samples nos. 1, 3, 4, 5, 6

Fig. 5. RT activity results for each PBMC sample: no. 1 (○—○); no. 2 (□—□); no. 3 (◆—◆); no. 4 (◇—◇); no. 5 (▲—▲); no. 6 (■—■); no. 7 (△—△); and no. 8 (●—●).

and 7 in the synthetic peptide test are shown in fig. 4. Nos. 1 and 5 were doubly infected by HIV-1 and HIV-2. Peptide tests were not needed for samples nos. 2 and 8, because clear cross-reactivities on core proteins were observed—HIV-1 p24 band for no. 2 (fig. 2) and HIV-2 p26 band for no. 8 (fig. 3).

Cell co-culture

An RT activity was obtained with four (50%) samples (fig. 5). The peak of RT activity was reached after 3 weeks of co-culture for cells from subjects nos. 5, 6 and 8, and 4 weeks for no. 3. The highest peak (340×10^3 cpm/ml) was observed with sample no. 8. However, weak RT activities of 10^3 – 20×10^3 cpm/ml, were detected with other samples. Only

HIV-1 reactivities were detected by the indirect immunofluorescence assay.

PCR studies

DNA extracted from PBMCs from each patient was amplified by PCR with all sets of primers. All the amplified DNA fragments were subjected to Southern blot analysis.

The amplified HTLV-I DNA fragment from sample no. 5 was the only one to yield a 188-bp band visible on an ethidium bromide-stained gel under UV light, as well as on Southern blot (fig. 6). Six (75%) DNA samples were amplified with at least one of the two HIV-1 pairs of primers; nos. 2 and 4 gave completely negative results (fig. 7). All DNA preparations gave

Fig. 6. Southern blot analysis of amplified HTLV-I DNA sequence obtained with primers 1, 2 in the *gag* region for the eight samples.

Fig. 7. Southern blot analysis of amplified HIV-1 DNA sequence for the eight samples obtained with (a) primers 3, 4 in the *gag* region, and (b) primers 5, 6 in the *pol* region.

Fig. 8. Southern blot analysis of amplified HIV-2 DNA sequence for eight samples obtained with (a) primers 7, 8 in the *pol* region, and (b) primers 9, 10 in the *env* region.

positive results with at least one pair of HIV-2 primers (fig. 8). Sample no. 6 showed a positive result with a 375-bp spot amplified in the HIV-2 *pol* region whereas

the Southern blot corresponding to the *env* amplified fragment was negative.

Overall results are summarised in table II.

Table II. HTLV-I, HIV-1 and HIV-2 infections characterised by culture, serological and PCR techniques on samples from eight patients

Patient no.	HTLV-I			HIV-1			HIV-2			Synthetic peptide test		Culture (RT)	Conclusions on retroviral infection
	ELISA	WB	PCR	ELISA	WB	PCR	ELISA	WB	PCR	1	2		
1	—	i	—	+	+	+	+	+	+	++	++	—	Mixed HIV-1/2
2	—	—	—	+	i	—	+	+	+	ND	ND	—	HIV-2 only
3	—	—	—	+	+	+	+	+	+	++	—	+	Mixed HIV-1/2
4	—	i	—	—	i	—	+	+	+	—	++	—	HIV-2 only
5	+	+	+	+	+	+	+	+	+	+	++	+	HTLV-I, HIV-1/2
6	—	i	—	+	+	+	+	i	+	++	—	+	HIV-1, HIV-2?
7	—	—	—	+	i	+	—	—	+	—	—	—	HIV-1, HIV-2?
8	—	i	—	+	+	+	+	i	+	ND	ND	+	HIV-1, HIV-2?

i, WB with an indeterminate interpretation; ND, not done.

Discussion

Mixed retroviral infections are often evoked during epidemiological surveys.^{7,8} However, these results are based only on serological data, and evidence of co-infection is rarely sought by culture^{15,16} or molecular analysis.¹⁷ Our samples from Ivorian patients had a high probability of showing such serological patterns. Several reasons may explain this phenomenon: firstly, the rate of AIDS progression in the Ivory Coast¹⁸; secondly, its geographical situation in West Africa where HIV-2 is more prevalent;¹⁸ and thirdly, the presence of HTLV-I infection in this area.⁸

In this study, a panel of techniques that allowed the precise diagnosis of these multiple infections was used. Investigative strategies usually include a highly sensitive ELISA screening test; the three ELISAs were found to be very sensitive. However, the HIV ELISAs showed non-specific reactions. This observation has been widely reported,¹⁹ especially with sera from African subjects. The presence of auto-antibodies, of circulating immune complexes and of elevated serum immunoglobulin levels, commonly found in tropical populations with heavy parasitic burdens, may account for these false positive results.^{19,20} Sera that gave indeterminate HTLV-I WB interpretations were negative by ELISA.

The rate of HIV-positive cultures in confirmed seropositive persons varies from 10 to 95%.²¹ No discrepant results were observed between cell co-culture and immunoserology. However, only HIV-1 isolates were obtained. The poor yield of HTLV-I in culture and the weaker cytopathic effect of HIV-2 may explain this result. Furthermore, weak RT activities detected, with four samples may correspond to slow/low HIV strains that grow slowly and yield low RT activity. These poorly replicating strains are often obtained from individuals with no or mild disease.²²

Immunoblot and PCR results for HTLV-I showed one clearly seropositive serum (no. 5). Wiktor *et al.*⁹ found that the intensity of the p24 band was usually greater than that of the p19 band in most HTLV-I

positive samples, whereas the opposite WB pattern was observed with HTLV-II positive samples. Since HTLV-II has been found to be more highly prevalent among certain populations such as intravenous drug abusers, numerous researchers have attempted to establish reliable tests to differentiate between HTLV-I and HTLV-II infections.^{9,23} Four sera produced an indeterminate interpretation on WB with reactivities to core proteins (fig. 1a), which is commonly observed with HTLV-I infection.²⁴ These bands might correspond to cross-reactions with other retroviruses, especially simian retroviruses.²⁵ Another non-specific band located at a higher mol. wt than the p53 band might be associated with contamination of the viral antigen preparation by HLA or HLA-like antigens from the producing cell line.²⁶

Two patients were HIV-2 positive (nos. 2 and 4) with an indeterminate HIV-1 WB. The presence of a p24 band alone (no. 2) may correspond to antigenic cross-reactivity between HIV-1 and HIV-2 *gag* proteins. Sample no. 4 showed three bands, one of which was a p31 band; this pattern strongly suggests a retroviral reactivity because *pol* regions are known to be highly conserved among retroviruses.²⁷ Unfortunately, we cannot follow up this subject to see if there was evolution of this WB pattern. Generally, these unusual WB patterns rarely change on follow-up testing.²¹

Two mixed HIV-1/HIV-2 infections (nos. 1 and 3) were detected. For sample no. 1, HIV-1 *gag* and HIV-2 *pol* fragments failed to be amplified at a primer annealing temperature of 53°C. Thus, PCR conditions that are too specific may lead to false negative results, especially with African samples that are known to have more genomic variability than European or American samples. We have already demonstrated that a lower annealing temperature allows a certain degree of sequence mismatch.¹¹ However, this protocol remains difficult for diagnostic purposes. This emphasises the necessity to use multiple primer pairs located in different regions of the HIV genome.²⁷ For sample no. 3, only the synthetic peptide test (fig. 4) was unable to show an HIV-2 positive infection. This type of

pattern was observed by Simon *et al.*²⁸ who suggest titration of antibody against specific peptides and comparison of titres for diagnosis of double infection.

We detected three ambiguous mixed HIV-1/HIV-2 infections (nos. 6, 7 and 8). However, nos. 6 and 8 showed a clear-cut HIV-1 positivity. The discrepancies observed between HIV-2 WB and PCR results did not resolve this problem. The indeterminate HIV-2 WB interpretations are probably associated with false positive PCR results.²⁷ Homologies between human retroviruses might explain this finding.²⁷ Moreover, Böttiger *et al.*²⁹ have demonstrated envelope protein cross-reactivity between HIV-1 and HIV-2. For subject no. 7, only the PCR results demonstrated a mixed HIV-1/HIV-2 infection. In this case, other tests such

as radio-immunoprecipitation assay or sequence analysis of PCR amplified DNA fragments are needed.

A triple retroviral infection (no. 5) was clearly demonstrated with each of the techniques. Bartholomew *et al.*³⁰ suggested that multiple-infected subjects may be at greater risk of developing AIDS. Thus, PCR analysis could provide new information in areas where the prevalence of HTLV-I/II, HIV-1 and HIV-2 is high.^{8,18}

Finally, these data obtained with "difficult samples" point to the need to develop and compare other virological tests for the detection of mixed retroviral infections. PCR could be a valuable additional technique for characterising these multiple retroviral infections.

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