A complex human immunodeficiency virus type 1 A/G/J recombinant virus isolated from a seronegative patient with AIDS from Benin, West Africa

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A human immunodeficiency virus type 1 (HIV-1 B76) originating from Benin (West Africa) was isolated and characterized. The patient had severe clinical AIDS and presented an unusual serological profile. Only one out of five different detection assays was able to demonstrate the presence of antibodies to HIV, whereas confirmatory assays remained indeterminate. In contrast, both plasma viral load and p24 antigen level were unusually high. HIV-1 infection was proved by viral RNA and proviral DNA amplification. HIV-1 B76 partially purified lysate reacted strongly with all anti-HIV-1-positive sera from the region but B76 plasma did not react with subtype A control viral antigen. This patient is likely to have had severe acquired immune dysfunction explaining her lack of immunological reactivity. Phylogenetic analysis of the genome identified a complex HIV-1 A/G/J recombinant. The gag and pol genes, and the majority of nef, are characteristic of subtype A; the gag/pol junction, the 3′ end of pol, vpu and env genes were characteristic of subtype G; vif, vpr and the 5′ end of nef were subtype J. In addition, part of the HIV-1 B76 genome had considerable sequence similarity with the previously described CRF06 cpx (BFP90) isolate. HIV-1 B76 did not exhibit any remarkable replication properties or cell tropism in vitro.

Introduction

Human immunodeficiency virus (HIV) infection is routinely diagnosed by detection of specific antibodies to HIV in serum or plasma. This technique, though effective in most cases, has shown limitations. After HIV exposure there is a 22–27 day window period before the development of a detectable antibody response (Busch et al., 1995). It has also been shown that distant HIV variants can remain undetected by commercially available assays, which are mainly manufactured with B subtype antigens (as was the case for HIV-1 groups O and N: Gurtler et al., 1996; Simon et al., 1998). In addition, a small number of HIV-immunosilent AIDS-progressing patients have been described (Montagnier et al., 1997; Sullivan et al., 1999; Ellenberger et al., 1999). Diagnosis in such cases can be made by detecting HIV antigen (p24 antigen EIA) or HIV genomic sequences (RT–PCR).

Retroviruses are highly recombinogenic, even when recombination can only occur between genomes packaged within the same virion (Robertson et al., 1997). It was initially thought that an individual could not be infected with multiple HIV-1 strains and recombination between divergent viruses did not contribute to HIV evolution. In fact, an increasing fraction of the published HIV-1 strains are intersubtype recombinants reviewed by Robertson et al., 1997, the first being Z321B, an A/G/I hybrid isolated in 1976 in Zaire (Choi et al., 1997; Gao et al., 1998b). It has been suggested that 10–60% of the viruses currently isolated may have mosaic genomes (Robertson et al., 1995; McCutchan et al., 1999). This may be an underestimate considering that most isolates have been only partially sequenced and that recombination can be missed if long portions of the genome are not analysed. The epidemiological significance of this fact is still unclear.

Currently, only six of the published HIV-1 recombinants are accepted as HIV-1 circulating recombinant forms (CRF; summarized by Carr et al., 1998a; Robertson et al., 1999; June 2000 report of Los Alamos HIV Sequence Database: CRF01 AE (CM240) virus from Asia, CRF02 AG (IbNG) from West
and Central Africa; CRF03 AB (Kal153) from Russia; CRF04 cpx (CY032) from Cyprus and Greece (Gao et al., 1998a; Robertson et al., 1999); CRF05 DF (V11310), though isolated in Belgium probably originated in the Democratic Republic of Congo (Laukkanen et al., 2000); and CRF06 cpx (BFP90) from West Africa (Oelrichs et al., 1998; Montavon et al., 1999). It is not known to what extent the generation of new complex recombinants may be related to the increasing prevalence of recombinant viruses. Sequential recombination events, in which an early recombinant serves as a parental strain for later ones, have been described (Salminen et al., 1997).

The present report describes a patient infected with a complex HIV-1 recombinant that escaped diagnosis, based on antibody detection, but was identified by molecular screening. The virus has been isolated and characterized. This is the second report of an HIV-1 recombinant infecting a nearly immnosilent host.

Methods

- **Patient selection and sample collection and storage.** Patient B76 was tested in the context of a collaborative study between the Centre National Hôpitalier Univeristaire in Cotonou (Benin), the Centre de Diagnostic Medical in Lomé, Togo, the Department of Microbiology, Komfo Anokye Teaching Hospital in Kumasi, Ghana and the University of Cambridge (UK). Fresh serum specimens obtained from 5781 patients with clinical AIDS were tested with two anti-HIV screening and CD4+ level assays [Enzymun-test Anti-HIV-1 + 2 + O, Boehringer Mannheim (BM)/Roche. Germany; ELAVIA Ac-Ab-Ak I. and Capcella, Sanofi-Pasteur, Marnes-la-Coquette, France]. The majority of patients (75.7%) were anti-HIV positive but 1404 (24.3%) were HIV seronegative and, after verbal informed consent, 40 ml blood samples were collected from the latter group in Vacutainer CPT tubes (Becton Dickinson). After separation, plasma and peripheral blood mononuclear cells (PBMC) were stored at −80 °C and sent to Cambridge for subsequent analyses. All HIV seronegative samples received were tested for HIV RNA by RT–PCR; patient B76 was one of three containing the HIV-1 genome and was studied further. The first such patient has been described previously (Candotti et al., 2000). Plasma from some HIV-seropositive patients was similarly recovered and processed for use as positive controls. Blood samples from HIV-seronegative British blood donors were obtained from the National Blood Service (Cambridge, UK).

- **Serological analysis.** The anti-HIV antibody analysis was performed using several commercial screening assays (Enzymun-test Anti-HIV-1 + 2 + O and Enzymun-test HIV combi, BM/Roche; ELAVIA Ac-Ab-Ak I, Sanofi-Pasteur; HIV-1.2.O GE94/95, Wellcozyme HIV Recombinant VK56 and GE22, Abbott/Murex, Darford, UK) as well as two confirmatory assays (HIV BLOT 2.2 Western blot, Genelabs Diagnostics, Singapore; INNO-LIA HIV confirmation, Innogenetics, Zwijndrecht, Belgium). In order to test B76 independently, an aliquot was sent to the Public Health Laboratory at Addenbrooke’s Hospital (Cambridge, UK), where three assays were performed (Vironostika HIV Uni-Form II plus O, Organon Teknika, UK; Serodia-HIV, Fugirebio Inc. and INNO-LIA HIV confirmation, Innogenetics). The p24 antigenemia in plasma was quantified using Murex HIV Antigen MAb (Abbott/Murex). Each assay was performed according to the manufacturer’s instructions. Results are expressed as sample-to-cut off ratio (S/CO). For acid dissociation, 100 µl of B76 positive and negative control plasmas were adjusted to pH 3.5 with 0.5 M HCl and heated for 60 min at 37 °C, then mixed with 100 µl sample diluent provided with Murex GE94/95, neutralized to pH 7 with 0.5 M NaOH, and immediately added to the coated microtitre wells provided with the assay. GE94/95 was performed simultaneously with treated and untreated plasma.

- **EIA using HIV crude viral lysate.** B76 positive and negative control crude viral lysates were prepared as previously described from SUPT-1 culture supernatant (Chaillyne et al., 1998; Candotti et al., 2000). Protein concentration was determined using BCA protein assay reagents (Pierce) and HIV antigen was quantified using Murex HIV Antigen MAb (Abbott/Murex). For each lysate, 2 µg of protein per well was coated on microtitre plates (Nunc Polysorb #44140A C96). ELISA was performed using Peptide HIV-1/HIV-2 EIA (Sanofi-Pasteur) on plasma samples diluted 1:50.

- **HIV isolation.** HIV was cultured from B76 plasma or PBMCs as previously described (Candotti et al., 2000). Virus replication was detected by measuring RT activity in culture supernatant using the Reverse Transcriptase Assay, Chemuluminiscent (BM/Roche). The replication properties of HIVp76 were tested by infecting cell lines possessing known HIV receptors and co-receptors. MT2 and SUPT-1 cells were used as described (Candotti et al., 2000). U87.CD4 glioma cells ARP072 and ARP073 stably expressing the chemokine receptors CCR5 and CXCR4, respectively, were infected according to Tscherning et al. (1998).

- **Molecular analysis** Viral particle-associated RT activity in plasma was detected by the procedure described by Pyra et al. (1994). HIV RNA load was estimated in plasma by bDNA hybridization (Quantiplex HIV, Chiron, Emeryville, CA, USA). Viral RNA was extracted from plasma using QIAamp Viral RNA (Qiagen). Reverse transcription was performed in 20 µl reactions containing 2.5 U MuLV-RT (PE Applied Biosystems), 1 U RNase inhibitor (PE Applied Biosystems), 0.08 A260 units of Random Primers (BM/Roche), and 1 mM of each dNTP (Amshem Pharmacia) (1 min at 25 °C, 60 min at 42 °C, 5 min at 95 °C). PCR amplifications were performed using AmpliTaq Gold DNA Polymerase (PE Applied Biosystems). Three sets of primers were used for the specific amplification of HIV-1, -2 or -O (BJgag1, -2 and -O; gag, CM-5/CAM3’ annealing in nef/3’LTR; Candotti et al., 2000; Coutlee et al., 1991; Maclere et al., 1997). Together with two sets of degenerate primers able to amplify all HIV-1, -2 and -O (GAG fwd, GAGrev and POlfwd/POlrev; Davies et al., 1999; Candotti et al., 2000). To amplify the V3–V4 region of the env gene, primers EST/ED33 were used (Delwart et al., 1995). For sequencing the whole B76 genome primers MSF12 and MSR5 (annealing to the tRNA-binding site in the 5’LTR and the mRNA polyadenylation site in the 3’LTR; Salminen et al., 1995) were added to the pool of primers already described. Overlapping fragments were amplified by long expand RT–PCR (Titan One Tube RT–PCR System, BM/Roche). Amplicons were sequenced using Thermo Sequenase dye terminator cycle sequencing pre-mix kit (Amersham Pharmacia) in an ABI 373 DNA Sequencer (PE Applied Biosystems). Four separate amplicons of each fragment were sequenced in both the forward and reverse directions and the sequences were compared and edited by hand. The consensus sequence was tested by translation for the appropriate HIV ORFs and the final sequence was used for analysis.

- **Phylogenetic analysis.** The B76 sequence was aligned using CLUSTAL W software (Thompson et al., 1994) with a series of HIV-1 subtype A–K subtype reference sequences from the Los Alamos HIV Sequence Database. Phylogenetic trees were constructed using the neighbour-joining and maximum likelihood methods included in the Phylib software package (Felsenstein, 1996). The reliability of the trees
HIV-1 recombinant in a seronegative patient

was estimated by bootstrap replications using Seqboot and Consensus included in the Phylip package. Additional similarity plots were performed using Simplot 2.5 (Ray, 1999) and the recombinant identification program (RIP). The identification of recombination breakpoints was done using the bootscanning method implemented in Simplot 2.5 and the RIP method, which enables more precise localization of recombination sites (Siepel & Korber, 1995).

Results

Clinical data

Patient B76 was a 32-year-old woman from Benin. She presented at the clinic in Cotonou with chronic diarrhoea of more than 3 months duration, fever over 38.5 °C for more than a month and oropharyngal candidiasis. Anti-HIV antibodies tested with two separate assays were negative (S CO 0 -6 and 0 -58). The CD4⁺ cell level was 36/µl.

After obtaining verbal informed consent, a second blood sample was collected to prepare plasma and PBMC. The patient died 1 week later.

Serological analysis

(a) Antibodies to HIV. B76 plasma was tested with four commercially available anti-HIV screening assays. As shown in Table 1, this plasma was not reactive with Enzymun HIV-1 +2 +O and ELAVIA EIAs, but was reactive with HIV-1.2.O GE94/95 (S CO 8 -18). B76 was also reactive with the Enzymun combi HIV Ag-Ab (S CO 7.6), which detects HIV antigen and antibodies. Consistent with this data, the p24 antigenemia was 7500 pg/ml. The sample was indeterminate with two confirmatory HIV-1/2 assays. Bands at gp120 and gp41 were detected with the INNO-LIA HIV line immunoassay, and bands at p17, p39 and gp120 with the HIV BLOT 2.2.

Independent serological results were obtained from the Public Health Laboratory at Addenbrooke's Hospital, Cambridge. B76 plasma was negative with the Serodia particle aggregation test and INNO-LIA immunoassay and only weakly positive with the Vironostika EIA (S CO 1.36). According to current diagnostic criteria, patient B76 would have been considered HIV seronegative.

The GE94/95 assay was then performed simultaneously on B76 plasma either untreated or submitted to low pH immune-complex dissociation. No increase in reactivity was observed (data not shown).

(b) Study of the reactivity obtained with Murex GE94/95. To investigate whether the assay reactivity was related to the test format or the antigen composition of the assay, B76 plasma was tested independently with three commercially available kits from the same company (GE95 HIV-1.2.O sandwich assay, Wellcozyme VK56 HIV-1 competitive assay and GE22 HIV-2 capture assay), as well as different combinations of solid phase/conjugate. The results obtained were independent of

<table>
<thead>
<tr>
<th>Test</th>
<th>Result</th>
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<tbody>
<tr>
<td>Symptoms</td>
<td>Chronic diarrhoea, fever, oropharyngal candidiasis</td>
</tr>
<tr>
<td>CD4 cell level/µl</td>
<td>36</td>
</tr>
<tr>
<td>BM HIV EIA</td>
<td>Negative (B 0.6; UK 0.4)</td>
</tr>
<tr>
<td>BM combi HIV Ag-Ab</td>
<td>Positive (7.6)</td>
</tr>
<tr>
<td>Sanofi HIV EIA</td>
<td>Negative (B 0.58; UK 0.12)</td>
</tr>
<tr>
<td>Murex HIV EIA</td>
<td>Positive (8-18)</td>
</tr>
<tr>
<td>Vironostika HIV</td>
<td>Positive (1-36)</td>
</tr>
<tr>
<td>Serodia-HIV</td>
<td>Negative</td>
</tr>
<tr>
<td>INNO-LIA HIV confirmation</td>
<td>Indeterminate gp120/gp41</td>
</tr>
<tr>
<td>Genelabs HIV confirmation</td>
<td>Indeterminate p17/p39/gp120</td>
</tr>
<tr>
<td>P24 antigen</td>
<td>7500 pg/ml</td>
</tr>
<tr>
<td>Plasma viral load</td>
<td>4.3 × 10⁷ RNA copies/ml</td>
</tr>
<tr>
<td>RT activity in plasma (Mg²⁺)</td>
<td>Positive (4.35)</td>
</tr>
<tr>
<td>RT activity in plasma (Mn²⁺)</td>
<td>Positive (4.12)</td>
</tr>
<tr>
<td>HIV-1-specific RT–PCR</td>
<td>Positive</td>
</tr>
<tr>
<td>HIV-2-specific RT–PCR</td>
<td>Negative</td>
</tr>
<tr>
<td>HIV-O-specific RT–PCR</td>
<td>Negative</td>
</tr>
<tr>
<td>Virus isolation from plasma in PBMC</td>
<td>Positive</td>
</tr>
<tr>
<td>Virus isolation from PBMC in PBMC</td>
<td>Positive</td>
</tr>
<tr>
<td>Virus isolation from PBMC in SUPT-1</td>
<td>Positive</td>
</tr>
<tr>
<td>Virus isolation from PBMC in MT-2</td>
<td>Negative</td>
</tr>
</tbody>
</table>
Table 2. Laboratory results for B76 plasma using different Murex reagents

All assays were performed according to the manufacturer’s instructions. Results from plasma of six HIV-seronegatives (NC), and two African HIV-seropositive patients (PC) were averaged and expressed as S/CO values. GE95 HIV-1.2.O is a sandwich assay. Wellcozyme VK56 is a competitive assay. GE22/HIV2 is a capture assay.

<table>
<thead>
<tr>
<th>Solid phase (antigen composition)</th>
<th>Conjugate (antigen composition)</th>
<th>S/CO</th>
</tr>
</thead>
<tbody>
<tr>
<td>GE94/95 HIV-1.2.O</td>
<td>GE95 HIV-1.2.O (HIV-1 p24/gp41 fusion protein, HIV-2 gp36 and HIV-O peptide)</td>
<td>4.545</td>
</tr>
<tr>
<td>GE22/HIV2</td>
<td>GE22/HIV2 (peptide derived from the gp41 Gnann region of HIV-2)</td>
<td>0.307</td>
</tr>
<tr>
<td>Wellcozyme VK56</td>
<td>Wellcozyme VK56 [anti-HIV(1B) gp41 human antibodies]</td>
<td>1.420</td>
</tr>
<tr>
<td>GE22/HIV2</td>
<td>GE22/HIV2 (peptide derived from the gp41 Gnann region of HIV-2)</td>
<td>0.213</td>
</tr>
</tbody>
</table>

the assay format. B76 plasma was invariably reactive when antigen combinations able to detect both anti-HIV-1 and 2 were used, but not when reagents detected only anti-HIV-2. S/CO values obtained with B76 plasma were consistently lower than those obtained with other African HIV-1-seropositive plasma samples used as positive controls (Table 2).

(c) Immunoreactivity of B76 plasma to autologous viral proteins.

To examine the possibility that the lack of B76 serological reaction might be strain-dependent, B76 and LAI (HIV-1 subtype B positive control) were grown in SUPT-1 cells. Supernatants from both cultures and supernatant from non-infected SUPT-1 cells (included as negative control) were subjected to ultracentrifugation; the pellets were lysed and coated separately on ELISA plates. These antigen preparations were used to test B76 and a panel of HIV-1 seropositive and -seronegative plasma samples from African patients. B76 plasma did not react with either LAI or B76 lysate. The other eight seropositive samples reacted with both antigen preparations (Table 3).

(d) Immune reaction to other viruses. B76 plasma contained antibodies to several common viruses, including rubella, parvovirus B19, hepatitis A, cytomegalovirus, herpes simplex, varicella-zoster and measles viruses. No antibodies to hepatitis B, hepatitis C, human T-lymphotropic or Epstein–Barr viruses were detected.

Table 3. Immunoreactivity of B76 plasma with autologous and control viral proteins

For each antigen preparation, 2 µg of protein per well was coated. The EIA was performed simultaneously for B76 and a panel of HIV-1 subtype A seropositive plasma samples (PC-1 to 8). All samples were diluted 1:50.

<table>
<thead>
<tr>
<th>Plasma sample</th>
<th>Anti-HIV EIA (S/CO)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B76 antigen</td>
</tr>
<tr>
<td>PC-1</td>
<td>4.69</td>
</tr>
<tr>
<td>PC-2</td>
<td>7.85</td>
</tr>
<tr>
<td>PC-3</td>
<td>5.71</td>
</tr>
<tr>
<td>PC-4</td>
<td>7.82</td>
</tr>
<tr>
<td>PC-5</td>
<td>4.07</td>
</tr>
<tr>
<td>PC-6</td>
<td>7.50</td>
</tr>
<tr>
<td>PC-7</td>
<td>4.91</td>
</tr>
<tr>
<td>PC-8</td>
<td>6.78</td>
</tr>
<tr>
<td>B76</td>
<td>0.25</td>
</tr>
</tbody>
</table>

Virological analysis

(a) Virus isolation and replication properties. Virus was isolated successfully from both patient PBMCs and plasma by coculture with PBMC from healthy blood donors. RT activity was detectable 5 days after inoculation in both cultures but no
cytopathic effect was observed. The isolate was designated HIV$_{B76}$. The kinetics of replication of this isolate was studied in activated CD8-depleted PBMCs from HIV-seronegative donors after infection with a standardized 50 TCID$_{50}$ inoculum of virus. A peak of RT activity (> 15 pg/μl) was observed 7 days after infection and HIV$_{B76}$ was classified as a rapid/high replicating virus. The ability of HIV$_{B76}$ to replicate in the T-cell lines SUPT-1 and MT-2 was then investigated. B76 replicated in the SUPT-1 cells, inducing transient formation of syncytia. In contrast, B76 failed to replicate in MT-2 cells and was considered to have a non-syncytia inducing phenotype (NSI).

The recombinant cell line ARP073, which expresses both CD4 and CCR5, could be infected with HIV$_{B76}$ but the ARP072 cell line, which expresses CD4 and CXCR4, could not. These results indicate that HIV$_{B76}$ can use CCR5 but not CXCR4 as co-receptor.

(b) Presence of RT activity in plasma. B76 plasma was filtered and ultracentrifuged, and the presence of RT activity associated with viral particles in the pellet was analysed using the PERT assay. RT activity was detectable in the presence of either Mg$^{2+}$ and Mn$^{2+}$ (average S/CO 4.35 and 4.12) (Table 1).

Sequencing and sequence analysis

(a) Presence of HIV RNA in plasma. HIV RNA was amplified using two sets of degenerate primers annealing within gag and pol, a set of env primers, and HIV-1 specific gag primers. No amplification was observed with HIV-2-LTR and HIV-O-gag specific primers. The HIV-1 plasma RNA load 1 week prior to death was 4.3 × 10$^7$ copies/ml.

(b) Sequencing of HIV$_{B76}$. The phylogenetic analysis of the partial gag, pol and env sequences revealed that the gag and pol fragments clustered within HIV-1 subtype A but env clustered with the G subtype (data not shown). The whole HIV$_{B76}$ genome was therefore sequenced using primers designed specifically from the obtained B76 sequences, with the sole exception of the LTR extremities. Each amplification/sequencing reaction was performed in at least four independent experiments. The neighbouring fragments overlapped each other by 100–200 nt, and always showed concordance between overlapping sequences.

(c) Sequencing of cloned PCR products. To confirm that patient B76 was infected with a single recombinant and not two distinct viruses of different genotype, two fragments of HIV$_{B76}$ (400 bp gag GAGFwd/GAGRev and 700 bp env ES7/ED33 products) were amplified, cloned and sequenced from plasma-derived RNA. For each region 11 clones were obtained, sequenced, aligned with the B76 consensus sequence and phylogenetically analysed. The sequences obtained from the clones were not more different from each other than from the consensus sequence (data not shown). Two of the gag-cloned sequences contained a stop codon. No evidence for coexistence of more than one HIV lineage was obtained.

(d) Identification of ORFs. The analysis of 8757 nt of the B76 sequence revealed that all the HIV-1 genes (gag, pol, vif, vpr, tat, rev, vpu, env and nef) were complete, without in-frame stop codons, major deletions, insertions or rearrangements. The first two ORFs (gag and pol) have the capability to encode precursor polyproteins of 499 and 1032 aa respectively, and overlap with each other by 196 nt. A variable region can be observed at the gag–pol overlapping region, in which most of the expected amino acids 32–46 are substituted compared to the HIV-1 references. The env ORF encodes an 838 aa precursor that contains 28 potential glycosylation sites. The gp120 V3 loop includes the consensus sequence His-Ile-Gly-Pro-Gly-Gln-Ala-Phe.

Among the auxiliary genes, vif encodes a 194 aa protein, which is 2 aa longer than the strains from the Los Alamos HIV Sequence Database, owing to a 6 nt insertion in position 184 (aa 61–62). The rev ORF does not show the expected stop codon present in sequences from other isolates, due to a point mutation, T to G, in the first position of the codon. A potential alternative TAG stop codon is located three codons downstream. The nef ORF potentially encodes a 219 aa protein, which includes a 36 nt insertion near the 5′ end of the gene compared to some reference HIV-1 strains (positions 76–111). This insertion corresponds to two partially overlapping duplications of 27 and 9 bases respectively (data not shown).

Phylogenetic analysis

(a) Subtype determination. Phylogenetic analysis was performed separately for each ORF. The sequences obtained for B76 were aligned with a series of A to K subtype reference sequences from the Los Alamos HIV-1 Sequence Database, as well as with several recombinant sequences including genotype A, and phylogenetic trees were constructed. The A/G recombinant virus described by this laboratory (Candotti et al., 2000) was also included in the analysis as CRF.02.G82. The B76 sequence clustered within subtype A for gag, pol and vif, the pol gene clustering near the CRF02 AG representatives (bootstrap value 98%). In contrast, vif and vpr clustered within subtype J and were closely related to the recently described A/G/J recombinant CRF06 cpx (BFP90) (Oelrichs et al., 1998; Montavon et al., 1999), with which they form a separate branch (100% bootstrap value). vpu and env clustered within subtype G, again close to BFP90 (bootstrap value 100%, data not shown). Therefore, B76 appears as a complex recombinant comprising fragments of A, G and J subtypes (Fig. 1a).

(b) Recombination site identification and phylogenetic analysis. Recombination analysis was done in parallel with Simplot 2.5 software (Ray, 1999) and the RIP program (Siepel & Korber, 1995). The overall Simplot results using reference sequences of
Fig. 1. For legend see facing page.
A, G and J subtypes rooted with the D subtype clearly indicated a preponderance of A subtype in the 5' end of the B76 genome and of the G subtype at the 3' end (Fig. 1b). A G fragment was present in the overlap region between the gag and pol genes, a 1000 nt long fragment of J subtype in the regulatory gene region and the end of the env gene, while the nef region was essentially of A subtype.

A more precise map of the crossover locations was provided by the RIP program. Taking the nucleotide number identification provided by RIP, the B76 sequence started at nt 696, corresponding with the beginning of the gag gene. Using this numbering, B76 crossover locations were at nt 2203 (A to G), 2392 (G to A), 4147 (A to G), 5067 (G to J), 6034 (J to G), 7756 (G to A), 8034 (A to G), 8555 (G to J) and 8664 (J to A). The insertion site of the first 5' G fragment corresponded to the overlap between the gag and pol genes; the second G fragment recombinated at the 5' end of the vif gene; the central J fragment spanned the 5' end of the vif gene and the 3' end of the vpr gene. All other recombination sites were not associated with the start or end of a gene.

To support the Simplot and RIP results, we constructed phylogenetic trees for nine genome fragments generated by the predicted recombination locations corresponding to nucleotides 696–2203, 2204–2391, 2392–4146, 4147–5066, 5067–e033, 6034–7755, 7756–8554, 8555–8663 and 8664–end of the B76 sequence (nt 9562). The B76 sequence fragments were compared to two or three reference sequences from each subtype and to six CRF 1, 2 and 3 complete sequences. Eight of these trees are presented in Fig. 2. In all cases, results were in agreement with the recombination map and were supported by high bootstrap values. CRF.02.G829 was the first recombinant HIV-1 isolated from a seronegative Ghanaian patient in our laboratory; the same means of analysis were applied and this isolate was compared to B76. The crossover locations and the fragment subtypes were similar to B76 in the 5’ end of the genome (nt 2190 (A to G), 2515 (G to A), 4228 (A to G), 5040 (G to A)) (data not shown). The 3’ end of the genome was also a mosaic of A and G subtype fragments with recombination sites at 6020 (A to G), 6332 (G to A), 8380 (A to G), 8760 (G to A), 9155 (G to A); the end of the sequence being subtype G. No subtype J fragment was present in the G829 sequence. This analysis was supported by the phylogenetic analysis (Fig. 2) showing that G829 was closely homologous to B76 in three of the four fragments in the 5’ end of the HIV-1 genomes.

We also compared the B76 genome with other published A/G recombinant complete sequences using the Simplot program (Fig. 1c, d). In the 5’ half of the genome (gag and pol genes), B76 was closely related to CRF.02.IBNG but in the 3’ half of the genome, except the nef region, B76 was even more closely related to the AGJ recombinant BFP90.

**Discussion**

Patient B76 presented at the clinic with symptoms of AIDS, but was diagnosed as HIV-seronegative in three different laboratories (one in Cotonou and two in Cambridge). Plasma from this patient was clearly reactive with only one of the five commercially available anti-HIV screening assays used; it was negative or low positive with the other four, and the presence of HIV antibodies could not be confirmed with two confirmatory assays. In contrast, HIV\_B76 was detected in plasma by a range of molecular techniques. Both viral load and p24 antigen level in plasma were unusually high (4.3 × 10⁷ RNA copies and 7500 pg/ml respectively), consistent with a very low CD4⁺ cell level (36/µl; Table 1). The patient died 1 week after the blood sample was obtained. These features are consistent with the observation that high viral load is predictive of rapid clinical progression and death (Mellors et al., 1996) and that the highest viral loads tend to be observed during the initial and the late phases of HIV infection (Kahn & Walker, 1998). The clinical and biological condition of patient B76 could not be explained by a generalized humoral immune dysfunction since antibodies to a series of common human viruses were detected, and survival to adulthood, in Africa, would be unlikely. Distant HIV variants may remain undetected by commercially available anti-HIV screening assays that rely mostly on B subtype antigens. Although HIV\_B76 is a complex recombinant of multiple HIV-1 subtypes, it was not an antigenically divergent strain. Indeed, HIV\_B76 antigens were recognized by antibodies in a panel of HIV-1 subtype A-seropositive African plasmas (Table 3).

Rare HIV-infected patients with clinical AIDS but repeatedly negative for HIV antibody screening have been described (Montagnier et al., 1997; Sullivan et al., 1999; Ellenberger et al., 1999; Candotti et al., 2000). Similar to B76, most of them had very low CD4⁺ counts (less than 100/µl) and relatively high viral loads (up to 8 × 10⁶ copies/ml). Some of these cases progressed rapidly to AIDS and death. When
Fig. 2. For legend facing page.
Fig. 2. Phylogenetic analysis of B76 and reference HIV-1 isolates from the Los Alamos Sequence Database. Reference sequences were aligned on the B76 fragments between recombination crossovers. (a) nt 696–2202; (b) nt 2203–2391; (c) 2392–4146; (d) 4147–5066; (e) 5067–6033; (f) 6034–7755; (g) 8555–8663; (h) 8664–9562. The unrooted trees were constructed using the neighbour-joining application included in the Phylip software. The scale is provided for each separate tree; 100 bootstrap values were indicated on the relevant nodes.
tested, infected sexual partners of these individuals were seropositive, suggesting that immunosilence was related to an immune dysfunction of the host rather than specific characteristics of the virus strains. However, information regarding duration of HIV infection in patient B76, or HIV infection in a sexual partner, was not available. In late stages of HIV-related disease high viral loads with adsorption of a large proportion of the HIV antibodies onto the viral antigens might decrease the detectability of antibodies by antigen capture. However, such complexes essentially affect antibodies to the gag proteins but not to envelope proteins (Allain et al., 1991; Ellenberger et al., 1999). In B76 plasma, low levels of gp120, gp41, p39 and p17 were detectable, suggesting an overall low level of antibody production. As only one of the screening assays clearly identified B76 as HIV seropositive, the potential role of assay format and antigen composition in the detection of anti-HIV antibody in B76 plasma was explored. Assays from one manufacturer reacted with B76 plasma. Among the reagents included in this assay, one was not from a B subtype but originated from a subtype prevalent in Africa.

The B76 isolate did not exhibit particular replication or tropism properties in vitro. The rapid rate of B76 isolation was certainly related to the high plasma viral load. Cellular viraemia was also likely to be high considering that, despite the low CD4+ cell count, HIV was rapidly isolated from the patient’s PBMCs. HIV strains with an SI phenotype are associated with disease progression and are generally isolated from patients with advanced AIDS. However, previous reports also indicated that NSI viruses, like B76, could be isolated from patients at the late stage of AIDS. The highly complex origin of the B76 genome did not seem to negatively or positively affect its replication properties and cell tropism compared to other HIV-1 strains.

The initial partial sequence of B76 indicated that this isolate was an A/G recombinant. The nearly full-length sequence of B76 uncovered a more complex mosaic genome including sequences belonging to A, G and J subtypes (Fig. 1a). Other authors have emphasized how important it is to obtain complete sequences of an HIV isolate to identify its subtypes. Published CRFs were often initially considered pure subtypes on the basis of partial sequencing. Isolate IbNG (CRF02 AG) was initially described as a new subtype A strain (Howard et al., 1994; Howard & Rasheed, 1996; Gao et al., 1996), CRF01 AE a new subtype called E (Gao et al., 1996), and CRF04 cpx as a new subtype I (Kostrikis et al., 1995). Once their complete sequences were obtained, all were found to be recombinants with different levels of complexity.

Seven points of recombination were detected along the HIV-B76 Genome. Some of the positions seem common to other recombinant viruses (gag–pol overlapping region, end of pol, along the auxiliary genes, end of env and beginning of nef), suggesting the existence of potential recombination hot spots. This could be related to locations in which the genome adopts secondary structures, or to regions where there is a certain level of sequence identity, favouring recombinations. Alternatively, it could imply that recombination in other regions of the genome does not generate viable progeny (Robertson et al., 1997). A third explanation might be recombination between common circulating forms of HIV-1.

B76 appears to be mostly of A subtype in the 5’ half of the genome, and G subtype in the 3’ half, interspaced with small fragments of J subtype (Fig. 1a, b). The presence of A and G fragments is not surprising, as they are the most prevalent subtypes in this geographical area (Workshop Report from the European Commission and the Joint United Programme on HIV/AIDS, 1997; Davies et al., 1999). In addition, G subtype env and pol genes and the J subtype auxiliary genes of B76 show considerable similarity with the previously described AGJ.AU.BFP90 isolate (Fig. 1c). The high level of similarity with CRF02 AG (IbGN) in most of the pol gene, as well as with the MAL isolate in the nef gene (Fig. 1d) should also be noted. High similarity between two isolates can suggest either that one of them may have been originated from the other through further recombination, or that they originated from an unknown common ancestor. It is believed that IbGN is becoming the most prevalent HIV-1 variant in West Africa (Andersson et al., 1999), and it is considered to be as old as some of the non-recombinant subtypes (Carr et al., 1998b). It is less clear how subtypes/isolates that do not seem to be prevalent in the population were able to recombine and appear as fragments of complex recombinants. To elucidate the potential replication advantage or other special characteristics increasing recombination rate/efficiency would require the study of more recombinant viruses. It is known that recombination generates genetic diversity and can lead to the generation of advantageous traits and removal of deleterious genes (Worobey & Holmes, 1999). Therefore some recombinants may have an evolutionary advantage. Should the prevalence of recombinant viruses increase, the opportunity for them to encounter other variants and recombine, generating more complex combinations, would also increase. Monitoring the frequency and the complexity of HIV-1 recombinants as well as the clinical status of patients they infect might be important to project the development of the AIDS epidemic.

We wish to thank Dr U. Desselberger from the Public Health Laboratory at Addenbrooke’s Hospital, Cambridge, where HIV quantification and antibody detection, as well as antibody detection to other than HIV were performed, and Dr J. Duncan from Abbott/Murex, who provided us with Murex reagents. This work was supported by a grant from Roche, Germany.

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Received 25 September 2000; Accepted 29 January 2001