Molecular cloning of two West African human immunodeficiency virus type 2 isolates that replicate well in macrophages: A Gambian isolate, from a patient with neurologic acquired immunodeficiency syndrome, and a highly divergent Ghanian isolate

(beer variants/serology/nucleotide sequence comparison/simian immunodeficiency virus)

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Abstract

Human immunodeficiency virus type 2 (HIV-2)-related viruses were isolated from a Gambian dying of exclusively neurological disease (HIV-2D2194) and from an asymptomatic Ghanian (HIV-2D2000). Both strains exhibited properties of HIV-1 biological subtype c: they grew slowly and induced few or no syncytia but eventually produced high levels of particle-associated reverse transcriptase in cultures of fresh peripheral blood lymphocytes, and they established stable infection of T-lymphoma (HUT-78) and monocyte (U937) cell lines. Each produced even higher levels of reverse transcriptase when fresh human monocytes/macrophages were used as target cells. The viruses were molecularly cloned after a single passage in culture, in order to minimize in vitro selection of subtypes present in vivo. Restriction-site analysis showed heterogeneity among each isolate. Nucleotide sequence analysis of a portion of the HIV-2D194 genome revealed that it is a member of the prototypic HIV-2 family, displaying 13% divergence versus HIV-2D2000 and HIV-2NMZ, as compared to 9% divergence between HIV-2ROD and HIV-2NMZ. In contrast, HIV-2D2000 is the most highly divergent HIV-2 strain yet described: it is equidistant in relation between the known HIV-2 strains and the simian immunodeficiency virus isolates from rhesus macaque monkeys (23-25% divergence).

Human immunodeficiency virus type 1 (HIV-1) is known to comprise a family of highly divergent members. They differ in biological properties such as cell tropism or replication efficiency in a given cell system (2-6), in antigenic determinants leading to different serological responses (7, 8), and in their nucleic acid sequence, often reflected in marked restriction-site polymorphism (3, 9, 10). Multiple variants have been demonstrated to exist within individual patients (2, 3, 10, 11).

Analyses of sera from West African patients with acquired immunodeficiency syndrome (AIDS) led to the discovery of a second HIV type in 1986, now called HIV-2 (12). Antigenic determinants of most HIV-2 proteins are not recognized by antibodies against HIV-1 (12, 13). In fact, HIV-2 is more closely related to the simian immunodeficiency virus from macaque (SIVmac) than to HIV-1 (1, 14), and crossreactive antibodies between HIV-2 and SIV can readily be demonstrated (12, 15). Several lines of evidence indicate that the HIV-2 family of viruses may be as divergent as the HIV-1 family (1, 16-18). Clinically, HIV-2 infections have been associated with typical AIDS (12, 13), purely neurological disease (19), and asymptomatic infections (20).

In the present study, we describe the biological characterization, molecular cloning, and partial DNA sequence analysis of two HIV-2-related strains isolated from patients originating from Gambia and Ghana, respectively (18, 19). Both strains were found to have unique biological and/or genetic properties compared to the HIV-2ROD prototype.

Materials and Methods

Virus Culture in Human Lymphocytes and Permanent Cell Lines. Primary isolation and cultivation of HIV-2 from patients' peripheral blood lymphocytes (PBLs) cocultured with human umbilical cord blood lymphocytes (CBLs) were done as described (2). For the establishment of permanent virus-producing cell lines, HUT-78 or U937 cells were cocultured with infected CBLs. The human monocytic cell line U937 and the T-cell lymphoma line HUT-78 (21) were obtained from the American Type Culture Collection.

Virus Culture in Freshly Isolated Human Monocytes/Macrophages. Fresh human mononuclear cells (MNCs) were prepared from blood donations (22) and cultivated in supplemented RPMI 1640 medium on hydrophobic membranes (23). MNCs (3 × 10⁸) were introduced into 10-ml bags and inoculated after 1 day with CBLs (5 × 10⁸) infected with HIV-2D194 or HIV-2D2000. Two days after infection, the cells were transferred to 24-well plates, where the nonadherent cells were quantitatively washed off. The adherent cells were further cultured in 1.3 ml of medium, and surface markers were monitored. Virus production by these cultures was monitored by reverse transcriptase (RT) assay.

RT Assay and Western Blot Analysis. Determination of RT activity and Western blot analysis were carried out as described (2, 24, 25). HIV glycoproteins were isolated from cell lysates by chromatography on lentil lectin-Sepharose 4B (Pharmacia). Elution was with 0.2 M methyl α-D-mannoside. For Western blots, 10 μg of this material was used per lane.

Southern Blot Analysis and Molecular Cloning of HIV-2 Isolates. DNA was extracted from CBLs that had been infected with viruses from the first in vitro passage (3). Southern blots were hybridized (26) in 30% formamide at 37°C with the 32P-labeled pK2-10BamA clone of SIV DNA (27). Filters were washed twice in 2x SSC/0.1% NaDod-

Abbreviations: CBL, cord blood lymphocyte; HIV, human immuno-

deficiency virus; MNC, mononuclear cell; PBL, peripheral blood lymphocyte; RT, reverse transcriptase; SIV, simian immunodeficiency virus.

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SO\textsubscript{4}/0.05\% Na\textsubscript{2}P\textsubscript{2}O\textsubscript{7} and twice in 1 \times SSC/0.1\% NaDodSO\textsubscript{4}/0.05\% NaP\textsubscript{2}O\textsubscript{7} (20 min per wash) at 50°C. (SSC is 0.15 M NaCl/0.015 M sodium citrate, pH 7.) Filters were exposed to Kodak XAR film with an intensifying screen for 12 hr at −70°C.

Genomic DNA, partially digested with Sau3A I to obtain fragments 9–20 kilobases (kb) in length, was ligated to BamHI-digested EMBL3A phage DNA (28), packaged, and propagated in Escherichia coli K803. From both libraries about 5 \times 10\textsuperscript{5} plaques were screened as described above.

**Sequencing.** Parts of the phage DNA inserts were sequenced by the dideoxy chain-termination method (29) using modified T7 DNA polymerase (30). Sequences were compiled and analyzed by using the Beckman MicroGenie program (31).

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**RESULTS**

**Serological Characterization.** To further analyze the variability within the HIV-2 family, virus isolates from 2 out of 24 HIV-2 patients we have investigated were studied in detail. Patient 194 was a Gambian who had developed exclusively neurological symptoms (19). He died of progressive multifocal leukoencephalopathy associated with JC virus infection. In contrast, patient 205 from Ghana was asymptomatic (18) at the time of virus isolation.

Western blot analysis revealed protein bands characteristic for HIV-2 (12, 15) when HIV-2D\textsubscript{205} (Fig. 1A) or HIV-2\textsubscript{194} (Fig. 1B and C) was used as antigen and reacted with either HIV-2 control serum or autologous serum. In contrast, HIV-1 control serum reacted faintly with gag and pol products (Fig. 1B) and failed to react with viral glycoproteins (Fig. 1C). Typical for HIV-2-infected individuals, sera from patients 194 and 205 failed to react with the envelope-derived glycoproteins gp120 and gp41 of HIV-1 (Fig. 1D). Interestingly, serum from patient 205 weakly recognized the envelope precursor molecule gp160 of HIV-1 and displayed a weak but detectable reaction to several other HIV-1 proteins.

**Growth Properties of HIV-2\textsubscript{194} and HIV-2\textsubscript{205} in Lymphocytes.** RT activity was detected 15 days after cocultivation of PBLs from patient 194 with CBLs, and a maximal RT activity of 6 \times 10\textsuperscript{4} cpm/ml of supernatant from 10\textsuperscript{6} cells was reached by 22 days. When PBLs from patient 205 were cocultivated with CBLs, RT was observed at 35 days, with a maximal value of about 5 \times 10\textsuperscript{7} cpm/ml on day 40.

By inoculation of CBLs with defined amounts of HIV-1 and monitoring of the cultures for RT levels and cytopathic effects, four different HIV-1 subtypes had been distinguished according to their growth properties (3). While HIV-2\textsubscript{ROD} behaved very similarly to the most frequent biological HIV-1 subtype a, the two newly isolated HIV-2 strains had properties most similar to HIV-1 subtype c (Table 1).

**Growth Properties of HIV-2\textsubscript{194} and HIV-2\textsubscript{205} in Monocytes/Macrophages.** Since HIV-2\textsubscript{205} was derived from a patient with exclusively neurological symptoms, its ability to grow in adherent monocytes/macrophages was analyzed. RT values were 1.5 \times 10\textsuperscript{7} cpm/ml of supernatant from 30,000 cells on day 15, reached a maximum of about 18 \times 10\textsuperscript{7} cpm/ml on day 33, and decreased to about 2.5 \times 10\textsuperscript{6} cpm/ml by day 54 (Fig. 2). Thus, compared to the maximum virus production by HIV-2\textsubscript{194}-infected lymphocytes, the infected macrophages produced 100–150 times more virus per cell in the peak phase of production. Similarly, HIV-2\textsubscript{ROD}-infected monocyte/macrophage cultures produced 10- to 50-fold higher levels of RT than HIV-2\textsubscript{194}-infected lymphocytes (data not shown). In parallel experiments a HIV-1 isolate of subtype b (see Table 1) and HIV-2\textsubscript{ROD} reached maxima of only about 0.12 \times 10\textsuperscript{5} cpm/ml per 30,000 cells (data not shown).

**Infection of Cell Lines with HIV-2\textsubscript{194} and HIV-2\textsubscript{205}.** Stably producing cell lines were established with both HIV-2\textsubscript{194} and HIV-2\textsubscript{205} in HUT-78 or U937; RT values reached 2–6 \times 10\textsuperscript{5} cpm/ml of supernatant.

**Electron Microscopy.** Examination of HIV-2\textsubscript{194}-infected CBLs by thin-section electron microscopy (32) showed multiple crescents of budding viruses (Fig. 3), suggesting a relatively slow assembly that correlates with slow growth in these cells.

![Fig. 1. Western blot analyses of the patients' sera and the corresponding virus isolates. (A) HIV-2\textsubscript{205} antigens reacted with autologous serum (lane 1) and serum from patient 194 (lane 2). (B) HIV-2\textsubscript{194} antigens reacted with negative control serum (lane 1), HIV-1-positive serum (lane 2), HIV-2-positive control serum (lane 3), autologous serum (lane 4), and serum from patient 205 (lane 5). (C) HIV-2\textsubscript{194} glycoproteins reacted with the sera as indicated in B. (D) HIV-2 Western blot strips (Bio-Rad) reacted with HIV-1 control serum (lane 1), normal serum (lane 2), serum from patient 194 (lane 3), and serum from patient 205 (lane 4). Molecular sizes are indicated in kilodaltons.](image-url)

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**Table 1.** Comparison of HIV-1 and HIV-2 subtypes as distinguished by growth in fresh CBLs

<table>
<thead>
<tr>
<th>HIV-1 biological subtype</th>
<th>Time to reach maximal RT, days</th>
<th>Maximal RT in supernatant, (cpm/ml) \times 10\textsuperscript{-5}</th>
<th>CPE*</th>
<th>HIV-2 strain</th>
<th>Time to reach maximal RT, days</th>
<th>Maximal RT in supernatant, (cpm/ml) \times 10\textsuperscript{-5}</th>
<th>CPE*</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>2–3</td>
<td>0.5–10</td>
<td>+++</td>
<td>ROD</td>
<td>2</td>
<td>2–5</td>
<td>+++</td>
</tr>
<tr>
<td>b</td>
<td>1–2</td>
<td>0.5–10</td>
<td>+</td>
<td>D194</td>
<td>4–5</td>
<td>2–5</td>
<td>+</td>
</tr>
<tr>
<td>c</td>
<td>5–7</td>
<td>0.5–10</td>
<td>+</td>
<td>D205</td>
<td>4–5</td>
<td>2–5</td>
<td>−</td>
</tr>
<tr>
<td>d</td>
<td>7–21</td>
<td>0.03–0.1</td>
<td>−</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

All experiments with the HIV-1 strains (ref. 3) as well as with the HIV-2 strains were done with sister cultures of CBLs from a single donor. Between donors, the variation was minimal and the differences between the subtypes were consistently observed. For infection with HIV strains, each isolate (2000–5000 cpm of RT activity, equivalent to about 100 infectious units of subtypes a and b as determined by titration on CBLs) was inoculated into 1 ml of cells (0.5–1 \times 10\textsuperscript{6} cells).

*Cytopathic effect: ++++, formation of giant syncytia, followed by death of culture; ++ +, formation of multiple syncytia involving 20–100 cells, followed by death of culture; +, occasional small syncytia, with cell survival for 1–2 weeks followed by death of the culture at the normal rate; −, no syncytia.
Restriction-site maps isolated from screened and respectively, HIV-2D194, nomic DNA these retroviruses. HIV-2D,94 and bridization 23.1- which corresponds to pK2-1OBamA the nucleotides 1831-9005 free of macrophage-specific antibody My 4 (Coulter). Thus, the cultures were free of T helper lymphocytes.

Southern Blot Analysis of Virus Isolates from Patients 194 and 205. Infection of patients 194 and 205 by a HIV-2-related virus was confirmed by Southern blot analysis (Fig. 4). Due to the homology between SIVMAC and HIV-2ROD (33), the pK2-10BamA clone of SIV DNA (27) was used as probe. It spans nucleotides 1831–9005 of cloned SIVK2-10 (27, 34, 35), which corresponds to SIVMAC-251 (36, 37). The strong hybridization signals (Fig. 4) established a genetic link between HIV-2D194 and HIV-2D205 and the SIVMAC/HIV-2 family of retroviruses. As has been observed in several HIV-1 isolates (3, 38), the most abundant form of viral DNA observed in these cells was unintegrated and linear.

Molecular Cloning and Restriction Site Analysis. Two genomic DNA libraries of CBLs infected with HIV-2D205 and HIV-2D194, respectively, were constructed in bacteriophage and screened with the SIVMAC probe. Four positive clones were isolated from HIV-2D205 and seven from HIV-2D194. Restriction-site maps of two HIV-2D205 clones and four

**Fig. 2.** Replication of HIV-2D194 in fresh human macrophages. RT activity of supernatants from two sister cultures was measured starting with day 15 after infection. According to their morphology, up to 96% of the cells were macrophages. All cells in the infected as well as in the uninfected control cultures were found negative for OKT4A (Ortho), and 80–90% of the cells were positive for the macrophage-specific antibody My 4 (Coulter). Thus, the cultures were free of T helper lymphocytes.

**Fig. 3.** Thin-section electron micrograph of a CBL infected with HIV-2D194, showing multiple budding structures at the cell membrane and a free particle of isolate HIV-2D194. (Bar = 100 nm.)

HIV-2D205 clones are shown in Fig. 5. Only clone HIV-2D194,5 contains a full-length proviral genome. Clone HIV-2D194,10 is likely derived from a circular intermediate. The clones from both isolates are markedly different from the HIV-2ROD prototype and from each other. Especially, the restriction maps of the HIV-2D205 clones have little similarity with that of HIV-2ROD. Sets of clones from the same patient revealed restriction-site polymorphisms, demonstrating that several genotypic variants existed in both patients.

**Sequence Analysis.** HIV-2D194,10 and HIV-2D205,7 were sequenced partially, and the sequences were aligned with each other as well as with those of HIV-2ROD (40), HIV-2NIHZ (41), SIVMAC-251 (SIVK678 in ref. 34), SIVAGM (42), and HIV-1BRU (43) (review of sequences in ref. 34). Table 2 shows the position of the aligned sequences relative to the HIV-2ROD genome, the extent of sequence homology, and the length of alignment. HIV-2D194,10 clearly belongs to the HIV-2 family based on its similarity to the HIV-2/SIVMAC viruses. The present data show 87.6% sequence identity to HIV-2ROD and to HIV-2NIHZ, 74.9% to SIVMAC, 57.3% to SIVAGM, and 55.0% to HIV-1BRU. For comparison, at the nucleotide level, HIV-2ROD is 90.1% identical to HIV-2NIHZ, 76.3% to SIVMAC, about 58% to SIVAGM, and about 57% to HIV-1BRU.

**Fig. 4.** Southern blots of DNA (10 μg per lane) from CBLs infected with HIV-2D205 and HIV-2D194. The probe was the 7.2-kilobase pK2-10BamA clone from SIVMAC-251. Molecular size markers were HindIII fragments of λ phage DNA (λHind); sizes (in kilobases) are indicated at left and right. (A) DNA of HIV-2D205-infected cells, digested with the indicated restriction enzymes or undigested. (B) DNA of HIV-2D205-infected cells, digested or undigested, and undigested pK2-10BamA (unlabelled probe) as control.
The factors that determine the clinical outcome of HIV infection are not clear but probably involve biological properties of the viruses infecting the individual as well as other environmental and genetic factors specific to the host (2–4, 6, 44–46). It is now widely recognized that apart from the CD4 T-lymphocytes, other very important target cells of HIV derive from the monocyte/macrophage cell lineage. They are thought to be responsible for the dissemination of the virus throughout the body (47, 48), for the latency, and for the killing of lymphocytes by antigen presentation (for review, see ref. 49).

**DISCUSSION**

We studied the replication of HIV-2\textsuperscript{D194} and HIV-2\textsuperscript{D205} in monocytes/macrophages by cocultivation of MNCs with CBLs infected with either strain. This procedure mimics to a certain extent the situation in vivo, whereby monocytes might get infected in the bloodstream and then migrate to the tissues where they differentiate further. With this protocol, a remarkably high replication of HIV-2\textsuperscript{D194} in macrophages as compared to lymphocytes became apparent. Since HIV-2\textsuperscript{D194} was derived from a patient with severe neurological disease, this finding supports the hypothesis that viruses that grow well in macrophages reach the brain at a relatively early stage of infection. However, HIV-2\textsuperscript{D205} also grew well in macrophages, while the patient was asymptomatic at the time of virus isolation. Since HIV-2\textsuperscript{DOD} did not grow well in macrophages, high replication in these cells is not a general feature of HIV-2 strains. Variation between HIV-2 strains was also found based on their growth in CBLs (Table 1).

At present, the borders between the families of HIV-1 and HIV-2 are defined by the absence of serological crossreaction in the envelope glycoproteins as well as by genetic markers such as the additional genes vpx in HIV-2 or vpu in HIV-1.

**Table 2.** Nucleotide sequence comparison of HIV-2\textsuperscript{D194,10}, HIV-2\textsuperscript{D205,7}, and other known HIV and SIV strains

<table>
<thead>
<tr>
<th>Positions corresponding to HIV-2\textsuperscript{DOD}</th>
<th>% sequence identity (length of sequence compared, nucleotides)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV-2\textsuperscript{D194,10}</td>
<td>HIV-2\textsuperscript{NHZ} HIV-2\textsuperscript{D194,10} SIV\textsuperscript{MAC-251} SIV\textsuperscript{AGM} HIV-1\textsuperscript{BRU}</td>
</tr>
<tr>
<td>1345–3850</td>
<td>87.2 (2506) 86.7 (2504) — 75.6 (2514) 61.0 (2526) 61.8 (2521)</td>
</tr>
<tr>
<td>4709–6360</td>
<td>86.8 (1661) 86.8 (1663) — 75.4 (1665) 50.3 (1660) 50.6 (1672)</td>
</tr>
<tr>
<td>8000–8574</td>
<td>84.8 (584) 85.4 (597) — 70.3 (597) 56.3 (590) 52.5 (627)</td>
</tr>
<tr>
<td>9053–773</td>
<td>91.1 (1220) 92.6 (1052)§ — 75.1 (1228) 56.8 (1225) 48.4 (1224)</td>
</tr>
<tr>
<td>Total</td>
<td>87.6 (5971) 87.6 (5816) — 74.9 (6004) 57.3 (6001) 55.0 (6044)</td>
</tr>
<tr>
<td>HIV-2\textsuperscript{D205,7}</td>
<td>5103–5626 6876–7030 6816–7030 6816–7030 6816–7030 6816–7030</td>
</tr>
<tr>
<td>1254–1690</td>
<td>83.1 (437) 82.8 (437) 81.6 (261) 79.6 (437) 66.4 (438) 64.8 (438)</td>
</tr>
<tr>
<td>5103–5626</td>
<td>71.9 (527) 69.6 (527) 71.2 (528) 70.1 (529) 40.3 (549)§ 48.8 (531)</td>
</tr>
<tr>
<td>6816–7030</td>
<td>79.5 (215) 79.1 (215) — 77.2 (215) 54.1 (222) 55.1 (227)</td>
</tr>
<tr>
<td>Total</td>
<td>77.4 (1179) 76.3 (1179) 74.7 (789) 74.9 (1181) 52.3 (1209) 55.9 (1196)</td>
</tr>
</tbody>
</table>

*For location on the genome, see Fig. 5.
†Sequence data for the HIV-2\textsuperscript{D194,10} regions listed in this table will appear in the EMBL/GenBank and DNA Data Bank of Japan (DDBJ) nucleotide sequence data bases under the accession number J04542.
‡Including unmatches due to different length of corresponding sequences.
§Has 229-base-pair deletion within U3 region/nef gene relative to HIV-2\textsuperscript{DOD}.
The borders between the more closely related HIV-2 and SIV\textsubscript{MAC} families are more difficult to define and are further obscured by the finding of a HIV-2-related strain presented here that, based on the available data, is genetically equidistant between the known HIV-2 strains and SIV\textsubscript{MAC} (sequence identity in both directions 75%) and therefore may even belong to a family of its own. Our findings point to greater diversity within the HIV-2 family than has been observed in HIV-1, which suggests that the HIV-2/SIV\textsubscript{MAC} family is evolutionarily older or diverges at a higher rate.

We thank J. I. Mullins for providing the SIV clone pK2-10BanA, L. Montagnier for the reference strain HIV-2\textsubscript{ROD}, H. W. Doerr for HIV-2 sera, and W. Brugger for characterization of the macrophages. We thank J. I. Mullins for many helpful suggestions on the manuscript and H. D. Brede for critical discussions. M. Landersz, S. Pfeffer, A. Seipp, and U. Rapp provided expert technical assistance.

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