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# Biological and genetic characteristics of HIV infections in Cameroon reveals dual group M and O infections and a correlation between SI-inducing phenotype of the predominant CRF02_AG variant and disease stage

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**Abstract**

In Yaounde, Cameroon, HIV-1 group-specific V3 serology on 1469 HIV-positive samples collected between 1996 and 2001 revealed that group O infections remained constant around 1% for 6 years. Only one group N sample was identified and 4.3% reacted with group M and O peptides. Although the sensitivity of the group-specific polymerase chain reaction (PCR) in two genomic regions was not optimal, we confirmed, in at least 6 of 49 (12.2%) dual O/M seropositive samples and in 1 of 9 group O samples, dual infection with group O and M viruses ($n = 4$) or with group O or M virus and an intergroup recombinant virus ($n = 3$). Partial *env* (V3–V5) sequences on a subset of 295 samples showed that at least eight subtypes and five circulating recombinant forms (CRFs) of HIV-1 group M co-circulate; more than 60% were CRF02_AG and 11% had discordant subtype/CRF designations between *env* and *gag*. Similarly as for subtype B, the proportion of syncytium-inducing strains increased when CD4 counts were low in CRF02_AG-infected patients. The V3-loop charge was significantly lower for non-syncytium-inducing strains than for syncytium-inducing strains but cannot be used as an individual marker to predict phenotype. The two predominant HIV-1 variants in Africa, CRF02_AG and subtype C, thus have different biological characteristics.


*Keywords:* HIV-1 variants; Cameroon; Intergroup recombinant; Dual infection; Syncytium-inducing phenotype

## Introduction

Phylogenetic analyses of numerous strains of HIV-1, isolated from diverse geographic origins, have revealed that they can be subdivided into groups (M, N, O), subtypes (A–D, F–H, J, K), sub-subtypes (A1, A2; F1, F2) and circulating recombinant forms (CRFs; CRF01–CRF14) (Robertson et al., 2000). It was soon recognized that HIV-1 strains from Cameroon show a remarkable genetic diversity, despite the initially low HIV prevalence. The three HIV-1 groups co-circulate and most of the known HIV-1 group M variants have been described in Cameroon. HIV-1 groups O and N were originally reported from this region and are endemic to Cameroon and neighboring countries (De Leys et al., 1990; Gurtler et al., 1994; Peeters et al., 1997; Simon et al., 1998; Vanden Haesevelde et al., 1994). Several unique and circulating recombinant forms have been described, and recombination between HIV-1 group O and M viruses has even been documented in Cameroon (Peeters et al., 1999a; Takehisa et al., 1999). Although, several studies have already reported the predominance of CRF02_AG in Cameroon, the simultaneous presence of many HIV variants

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leads to the emergence of recombinant strains involving divergent strains (Carr et al., 2001; Fonjungo et al., 2000; 2002; Heyndrickx et al., 2000; Montavon et al., 2000; Nyambi et al., 2002; Tebit et al., 2002). Therefore, it remains important to continue to genetically characterize prevalent HIV-1 strains in regions where multiple variants co-circulate.

Continuous monitoring of the molecular epidemiology of HIV-1 is also necessary because of all the implications related to this diversity. There is well-established evidence that differences related to efficiency of diagnostic assays and antiretroviral drugs exist among the various HIV-1 variants (Apetrei et al., 1996, 1998; Descamps et al., 1997, 1998; Loussert-Ajaka et al., 1994; Parekh et al., 1999). The degree to which vaccines based on one subtype will elicit cross-protection against other subtypes is also still poorly understood but the discovery of large numbers of recombinant viruses clearly implies that co-infection with divergent HIV-1 strains is not as rare as once thought (McCutchan, 2000; Peeters, 2000; van der Groen et al., 1998). Recent studies have shown that there may also be differences in pathogenesis among certain subtypes (Kaleebu et al., 2002; Kanki et al., 1999).

Only a few data are available on the biological characteristics of non-B HIV-1 strains, but from several independent studies, it is clear that the CXCR4-positive phenotype, previously known as rapid/high or syncytium-inducing (SI), is rare among subtype C-infected patients (Bjorndal et al., 1999; Peeters et al., 1999b; Tscherning et al., 1998). For subtype B, the presence of CXCR4-positive viruses is associated with a more rapid progression to AIDS (Fenyo et al., 1988; Karlsson et al., 1994; Koot et al., 1992, 1993; Tersmette et al., 1988, 1989), but it remains to be determined whether subtype C infections are associated with different clinical outcomes. The majority of HIV infections worldwide occur in Africa where all HIV-1 variants co-circulate, but with subtype C predominating in South and East Africa and CRF02_AG in West and West Central Africa (McCutchan, 2000; Peeters and Sharp, 2000). Since CRF02_AG is the second predominant HIV-1 variant in Africa, it is important to gain better knowledge of its biological characteristics.

In this study, we report on the genetic and biological characteristics of HIV-1 strains in a large cohort of patients attending PRESICA, a health structure providing counselling, voluntary testing, and medical care for HIV-infected patients in Yaounde, the capital city of Cameroon, between 1996 and 2001.

## Results

### *Serologic prevalence of HIV-1 group M, N, and O and HIV-2 infection among HIV-positive patients between 1996 and 2001*

Over a 6-year period, from 1996 to 2001, 1469 samples, identified as HIV positive by commercial screening and confirmatory assays, were tested in an indirect ELISA based on synthetic V3-loop peptides to discriminate between HIV-1 group M, N, and O and HIV-2 infection. Overall, 1234 (84.0%; 95% confidence interval (CI), 82.1–85.9%) samples were identified as group M, 15 (1.0%; CI, 0.6–1.7%) samples were group O, 63 (4.3%; CI, 3.3–5.5%) samples reacted with group M and group O V3-loop peptides, and only 1 (0.1%; CI, 0.0–0.4%) sample reacted strongly with group N. Only 3 (0.2%; CI, 0.0–0.6%) samples were identified as HIV-2, and 153 (10.4%; CI, 8.9–12.1%) of the samples did not react with any of the V3-loop peptides used. The proportion of group M infections fluctuated from 1996 to 2001 between 78 and 90%, but differences were not significant over time ($\chi^2$ for trends, $P = 0.8$). Group O infections remained constant around 1% (0.8–1.6%) within the limits of the CI, and about 10% (7.9–11.7%) of the HIV infections could not be typed at each period. The proportion of samples that reacted simultaneously with group O and M peptides fluctuated over time, but no significant trend was seen ($\chi^2$ for trends, $P = 0.9$).

### *HIV-1 group M variants*

A subset of the 1469 patients ($n = 295$) agreed to be enrolled in a previously described cohort study that monitors whether different subtypes are associated with different disease progression or pathogenesis (Laurent et al., 2002). Samples from these patients were genetically characterized and can be considered representative of the complete HIV-infected population, without any biases in the subtype distributions. The genetic subtype was identified in the *env* V3–V5 region for these 295 samples, and for 118 of them, chosen at random, the subtype was also characterized in the *gag* p24 region. Among the 295 samples, 278 were reactive with the group M V3 peptide, and 17 were untypable in the peptide ELISA. Fig. 1 summarizes the genetic subtypes in the *env* V3–V5 region for the 295 samples. Overall, at least eight subtypes and five CRFs co-circulate in HIV-1 group M-infected patients in Yaounde. The majority of the strains (80.8%) belonged to subtype A, but a more detailed phylogenetic tree analysis showed that many strains formed a separate subcluster with the CRF02_AG prototype strains, representing 62.7% of the total amount of HIV-1 infections. Among the remaining *env* subtype A samples, some clustered with CRF11_cpx, CRF13_cpx, or the A reference strains, and a separate subcluster that we identified for clarity as A* was also observed (Fig. 2a), representing 4.8, 0.3, 8.2, and 4.8% of the total infections, respectively (Fig. 1). Almost all other known genetic subtypes co-circulated: 0.3% B, 0.3% C, 4.4% D, 0.3% F1, 6.4% F2, 3.1% G, 2.1% H, 0.3% K; two other CRFs were also present, 0.3% CRF01_AE and 1.7% CRF06_cpx (Fig. 1). Only one subtype F strain belonged to the F1 sub-subtype; all the others were F2. The majority of the *env* subtype D strains formed a separate and well-supported subcluster within subtype D (Fig. 2a). Previously described unusual *env* sequences from

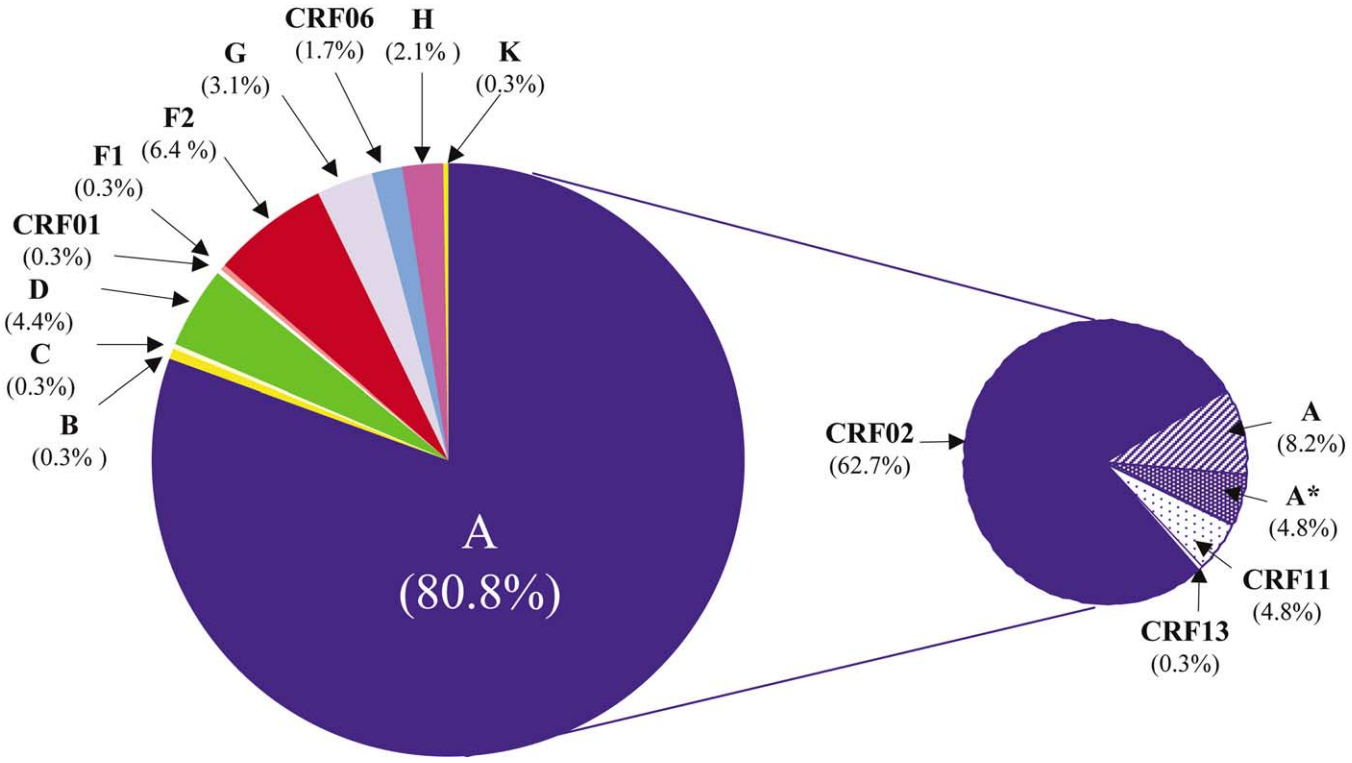


Fig. 1. Distribution of HIV-1 group M variants (in percentage) in Yaounde, the capital city of Cameroon. The data are based on partial *env* (V3–V5) sequences.

Cameroon (Cam001C and BCB88), initially proposed to form a new *env* clade designated "k" (Loussert-Ajaka et al., 1998; Roques et al., 1999), also clustered with our subtype D viruses. Similar subtype D variants were recently also documented by our group in Chad, and full-length genomes were sequenced for two such strains (99TCD-MN011 and 99TCD-MN012) showing that these strains represent a pure subtype D variant restricted to West Central Africa (submitted for publication).

The genetic subtype was also identified in 17 samples that were untypable by V3-group specific serology (17 of 153 untypables, 11.1%); all 17 were group M and the subtype distribution was as follows: 9 CRF02_AG, 1 A, 2 D, 1 H, 2 F2, and 2 CRF11. No significant differences were observed in the V3-loop sequences of these samples compared with the V3-consensus sequences for each subtype.

For 118 samples subtyped in *env*, we also genetically characterized the *gag* p24 region to estimate the proportion of recombinant strains. For 28 (24%) and 78 (66%) strains, subtype and CRF designations, respectively, were concordant between the two genes studied (Table 1). For 12 (10%) samples, the subtype/CRF designations were discordant between the two genes studied (Table 1). For 2 of the 12 discordant samples, two different subtypes were noted, but for the remaining 10 strains, the discordance was noted between a CRF and a subtype. Similarly as observed among the *env* sequences, *gag* subtype D sequences formed a separate subcluster within subtype D and the *env* subtype A sequences, designated as A* in *env*, clustered in the *gag* region with the CRF01_AE prototype strains (Fig. 2b). This cluster also contained the CM53122 strain from Cameroon, for which full-length genome analysis revealed

Fig. 2. Identification of new subclusters in subtypes A and D: phylogenetic trees based on 499-bp unambiguously aligned nucleotides from the V3–V5 *env* region (a) and 669-bp unambiguously aligned nucleotides from the p24 *gag* region (b). The following reference strains representing the different subtypes and CRFs were used: subtype A (A_KE.93.Q2317, A_UG.92.UG307, A_SE.94.SE7253); subtype B (B_US.86.JRFL, B_US.90.WEAU160, B_FR.83.HXB2R); subtype C (C_ET.86.ETH2220, C_IN.95.21068, C_BW.96.BW0502, C_BR.92.BR025); subtype D (D_CD.83.NDK, D_CD.83.ELI, D_UG.94.UG114, D_ 99TCD.MN011, D_99TCD.MN012); sub-subtype F1 (F1_FR.96.MP411, F1_BE.93.BEVI850, F1_FI.93.FIN9363); sub-subtype F2 (F2_CM.95.MP255, F2_CM.95.MP257); subtype G (G_NG.92NG083, G_SE.93.SE6165, G_BE.96.DRCBL); subtype H (H_BE.93.VI991, H_BE.93.VI997, H_CF.90.CF056); subtype J (J_SE.93.SE7887, J_SE.94.SE7022); subtype K (K_CM.96.MP535, K_CD.97.EQTB11); CRF01_AE (AE_TH.93.TH253, AE_TH.90.CM240, AE_CF.90.CF402, AE_CF.90.CF4071); CRF02_AG (AG_IBNG, AG_FR.91.DJ263, AG_CM.97.MP807, AG_SE.98.MP1211, AG_SE.98.MP1213); CRF06_cpx (96.BFP90, 95.ML84, 95.ML127), and CRF11_cpx (97.CM-MP818, 99.FR-MP1298, 99.FR-MP1307). The phylogenetic analysis was done as described under Materials and Methods. Reference strains are in black; our strains forming an A* subcluster and a $D^{CM}$ subcluster (D in Cameroon) are in gray. The CM53122 strain (marked by ▲) from Cameroon has been considered as a reference of CRF01_AE/A recombinant (Carr et al., 2001) and clustered with our group A*.

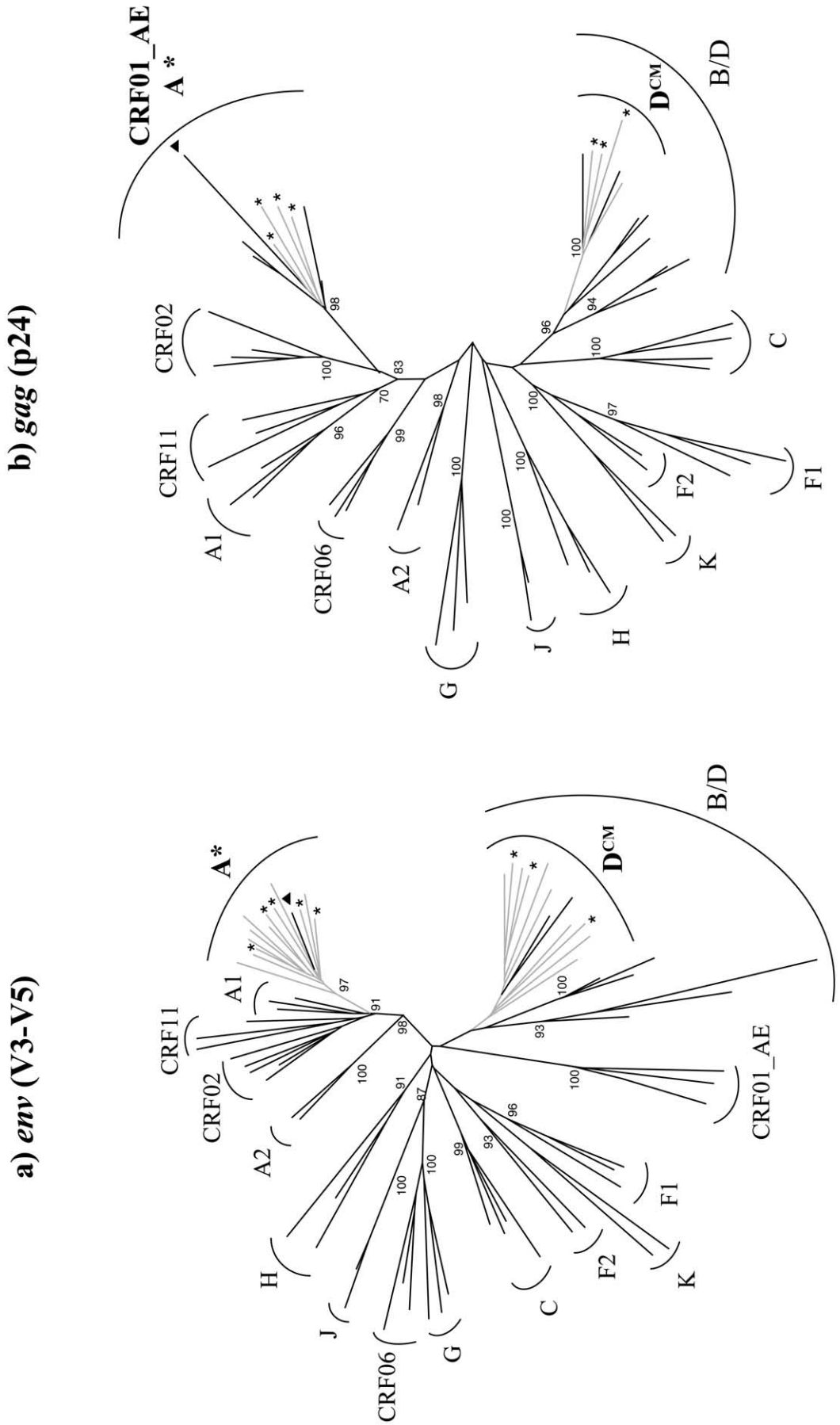
a) env (V3-V5)
A*
A1
CRF11
CRF02
A2
H
J
CRF06
G
C
F2
K
F1
D^CM
B/D
CRF01_AE
0.1
b) gag (p24)
CRF01_AE
A*
CRF02
CRF11
A1
CRF06
A2
G
J
H
K
F2
F1
C
D^CM
B/D
0.01

Table 1
Subtypes in *gag* (p24) and *env* (V3–V5) for 118 HIV-1 group M samples

| | *gag* | *env* | *n* |
|---|---|---|---|
| Concordant genotypes between *gag* and *env* | | | |
| Concordant subtypes (24%) | A | A | 9 |
| | B | B | 1 |
| | D | D | 3 |
| | F2 | F2 | 10 |
| | K | K | 1 |
| | G | G | 4 |
| Concordant CRFs (66%) | CRF02 (A) | CRF02 (A) | 69 |
| | CRF06 (A) | CRF06 (G) | 1 |
| | CRF11 (A) | CRF11 (A) | 7 |
| | CRF13 (G) | CRF13 (A) | 1 |
| Discordant genotypes between *gag* and *env* | | | |
| Discordant subtype/CRF designations (10%) | A | H | 1 |
| | D | A | 1 |
| | CRF02 | A* | 1 |
| | A | CRF02 | 1 |
| | CRF02 | H | 1 |
| | CRF02 | F2 | 1 |
| | CRF01 | A* | 6[a] |

[a] This group of viruses could represent a new CRF.

that it was a CRF01_AE/A recombinant strain (Carr et al., 2001).

### *Genetic characterization of dual group O and M seropositive samples*

Among the 1469 sera tested in the V3-loop ELISA, 63 (4.3%) reacted simultaneously with HIV-1 group M and group O V3 peptides. In 49 samples, for which sufficient material was available, additional PCR analyses with specific group M and group O primers in *pol* (partial integrase) and *env* (C2-V3 and/or partial gp41) were done to study whether these individuals were infected with both group M and group O viruses. Each PCR product was sequenced to verify the specificity of the group-specific primers. As controls, we performed similar analyses on 9 samples that reacted only with group O V3 peptides and on 10 samples that reacted only with group M V3 peptides. The dual-reactive samples were classified into three categories, according to the optical density (OD) observed with each peptide. Table 2 summarizes the PCR results for the different categories of dual-reactive samples and for the group O and group M control samples. Among the 34 samples with higher reactivity with the M-V3 peptide, only 1 dual infection was documented by PCR. In this sample (MP977), O and M viruses were amplified in *pol* and *env*. Both O and M viruses were confirmed by sequence and phylogenetic tree analysis of the C2-V3 region (Fig. 3). For the 14 samples with equal reactivities to group M and O peptides, 8 were infected with a group M virus only, 1 with a group O virus only; in 2 patients (MP647, MP1289), O and M sequences were amplified in *pol* and *env* (Fig. 3); and in 2 others (283BA, MP978), only M sequences were amplified in *pol* and O and M sequences in *env* (Fig. 3), suggesting co-existence of a group M virus and a possible intergroup recombinant virus. One of these samples was from a patient for whom we previously documented dual infection with group M and an intergroup M–O strain, based on full-length genome sequencing (Peeters et al., 1999a). The intergroup recombinant strain was M in the 5′ end of the genome and O in the 3′ end, with a breakpoint in *vif*. In the only sample with a higher reactivity with the O-V3 peptide (2734BA), both group O and M sequences were amplified in *pol* and *env* (Fig. 3). For two dual-reactive samples, no conclusion could be drawn because no virus could be amplified in *pol* or *env* with the group O- and M-specific primers.

Surprisingly, for one of the nine samples reacting with only the group O V3 peptide (273BA), the presence of at least two viruses was documented. In this sample, M and O were amplified in *env* (Fig. 3) and only O in *pol*. This could suggest the presence of dual infection with a group O virus and an intergroup recombinant virus being O in the 5′ end of the genome and M in the 3′ end. For two group O samples, no conclusion could be drawn because no virus could be amplified in *pol* or *env* with the group O- and M-specific primers.

For the 10 samples that reacted only with group M peptides, only group M sequences were amplified. Fig. 3 shows the phylogenetic tree in the *env* C2-V3 region confirming the simultaneous presence of group O and M sequences. Overall, this means that at least in 6 of 49 (12.2%) dual-seropositive samples, dual infection was documented, as it was in 1 of 9 (11.1%) group O monoreactive samples.

### *Syncytium-inducing capacity of non-B HIV-1 group M strains in Cameroon*

Classification of viral isolates as SI or non-SI (NSI) is considered to be important for the prognosis of disease progression, and the appearance of SI variants is related to enhanced CD4+ cell depletion and disease progression to AIDS in subtype B infections. Few data exist on the biological characteristics of CRF02_AG strains which are predominant in the HIV epidemic in West and West Central Africa. For the patients included in the cohort, viral cultures were attempted. For 168 randomly chosen HIV-1 strains the SI capacity was also studied on MT-2 cells as previously described. For the different subtypes, data were correlated to disease stage of the patients as reflected by their CD4 counts. Table 3 summarizes the number and/or percentage of SI strains, according to the CD4 counts and for the different *env* subtypes. Similarly as observed in subtype B-infected patients, more SI strains are observed in patients at a more advanced disease stage: 54.7% SI strains in patients with fewer than 200 CD4 cells/mm$^3$, 27.5% SI strains at CD4 counts between 200 and 500 cells/mm$^3$, and 12.5% SI strains at CD4 counts above 500 cells/mm$^3$. Only for the CRF02_AG strains, which represent 61.3% of the

Table 2
PCR results with specific group O and M primers in *pol* and *env* (C2V3) for the different categories of dual O/M seropositive samples and for the group O and group M control samples

| | Dual reactive samples ($n$ = 49) | | | Group O samples[d] ($n$ = 9) | Group M samples[e] ($n$ = 10) | Total |
|---|---|---|---|---|---|---|
| | OD O > M[a] ($n$ = 1) | OD M = O[b] ($n$ = 14) | OD M > O[c] ($n$ = 34) | | | |
| *pol* M, *env* M | — | 8 | 32 | — | 10 | 50 |
| *pol* O, *env* O | — | 1 | — | 6 | — | 7 |
| *pol* M+O, *env* M+O[f] | 1 | 2 | 1 | — | — | 4 |
| *pol* M, *env* M+O[g] | — | 2[h] | — | — | — | 2 |
| *pol* O, *env* M+O[g] | — | — | — | 1 | — | 1 |
| PCR neg with M and O in one of the two genes | — | 1 | 1 | 2 | — | 4 |

[a] OD with M-V3 peptide = 0.605 and OD with O-V3 peptide = 2.869.
[b] Mean OD with M-V3 peptide = 0.997 (0.386–1.687) and mean OD with O-V3 peptide = 0.950 (0.313–1.624).
[c] Mean OD with M-V3 peptide = 1.181 (0.703–2.283) and mean OD with O-V3 peptide = 0.538 (0.371–1.077).
[d] OD with M-V3 peptide < 0.250 and mean OD with O-V3 peptide = 0.947 (0.345–1.546)
[e] Mean OD with M-V3 peptide = 0.699 (0.374–1.205) and OD with O-V3 peptide < 0.250.
[f] These combinations of PCR results suggest infection with a group O virus and a group M virus.
[g] These combinations of PCR results suggest infection with a group M or O virus and an intergroup recombinant virus.
[h] Based on full-length genome sequence for one sample, an intergroup O/M recombinant virus has been previously reported on a follow-up sample from this patient (Peeters et al., 1999a).

strains tested, could significant conclusions related to the subtype be drawn; for the other variants the numbers tested were too low: 55.8% of the CRF02_AG strains were SI in patients with fewer than 200 CD4 cells/mm$^3$, 20.0% were SI when CD4 counts were between 200 and 500 cells/mm$^3$, and 20.0% were SI when CD4s were above 500 cells/mm$^3$.

Similarly as previously reported for subtype B strains, we studied the prediction of the phenotype based on the sequence of the V3 loop; i.e., we examined whether positively charged amino acids at positions 11 and/or 25 in the V3 loop were present, and calculated the global charge of the V3 loop (Resch et al., 2001). All V3-based phenotype predictions are summarized in Table 4. Among the 108 NSI strains in vitro, 103 (95.4%) were predicted as NSI and 5 (4.6%) were predicted as SI based on the presence of a positive charged amino acid at position 11 or 25, with R ($n$ = 3) and K ($n$ = 2) in position 25 only. For the 60 SI strains in vitro, only 20 (33.3%) had a positive amino acid at one of these two positions, with R ($n$ = 10) and H ($n$ = 2) at position 11 and with R ($n$ = 4) and *K* (n = 5) at position 25.

Only a significant number of CRF02_AG strains were analyzed and a similar trend was seen: 64 (95.5%) of the 67 NSI strains were predicted to be NSI and only 12 (33.3%) of the 36 SI strains were predicted to be SI. Although the numbers of subtype D samples were very low, this was the only subtype in our study where all SI strains were predicted SI based on the amino acids present at positions 11 and/or 25.

The mean global charge of the V3 loop was significantly lower for NSI strains, 4.25 (± 0.88), versus SI strains, 5.40 ± 1.30 ($P < 10^{-4}$). This was also the case for CRF02_AG separately, 4.16 ± 0.91 for NSI strains versus 5.42 ± 1.36 for SI strains ($P < 10^{-4}$). For subtype B, NSI strains have a total net charge between +2 and +4 and SI strains have a charge superior to +4. However, the global charge cannot be used as an individual marker to predict whether strains are SI or NSI, since only 66 of 108 (61%) NSI strains have a charge ≤4 predicting NSI strains for subtype B, and 45 of 60 (75%) have a charge ≥5 which predicts SI strains for subtype B (Table 4). Predicting the phenotype based on the global V3 charge was more sensitive than the method based on positively charged amino acids at position 11 and/or 25 (75% vs 33.3%) but less specific (61.1% vs 95.4%) (Table 4). These two methods were established on subtype B, with a better performance (sensitivity: 83% vs 84%; specificity: 93% vs 97%) (Resch et al., 2001) and cannot be used on non-B strains.

## Discussion

In addition to previous studies on molecular epidemiology in Cameroon, this current study highlights the prevalences of groups M, N, and O over time, documents dual group M and O infection and intergroup recombinants, and describes biological characteristics of the predominant CRF02_AG variant.

HIV-1 groups O and N were both first identified in Cameroon, raising concern over the emergence of new HIV variants, with possible public health implications (Loussert-Ajaka et al., 1994). In this study, we showed that group O prevalences remained low and constant, representing around 1% of the HIV-1 infections between 1996 and 2001. Previously, Ayouba et al. (2001) showed, for samples collected between 1986 and 1998, that group O infections decreased in Cameroon from 20.6% in 1988 to 1.4% in 1998. These data and our subsequent stable prevalences suggest that group O infection seems to be stabilized. We

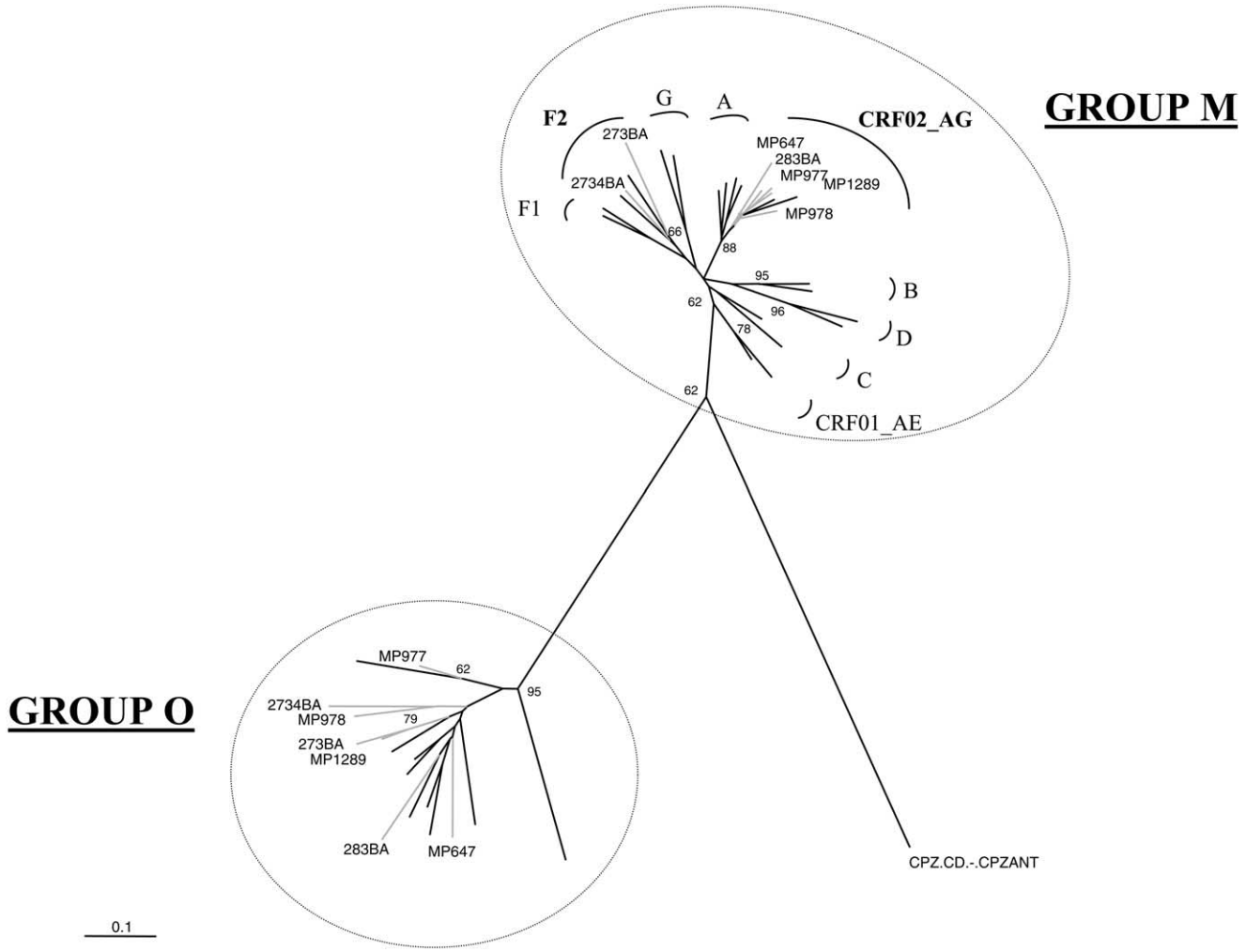


Fig. 3. Phylogenetic tree based on 364-bp aligned nucleotides from the C2V3 *env* region for the dual seropositive samples in which group O and M sequences were amplified in *env*. The following reference strains representing group O strains and different group M subtypes and CRFs are as follows: O_CM.ANT70, O_GQ.276HA, O_GA.92.VI686, O_SN.99.SEMP1299, O_SN.99.SEMP1300, O_GQ.193HA, O_CM.93.CA9EN, O_CM.91.MVP5180, O_CM.4974; A_KE.93.Q2317, A_UG.92.UG307, A_SE.94.SE7253, A_SE.94.SE7535, B_GA.88.OYI, B_FR.83.HXB2R, C_BW.96.BW0402, C_BR.92.BR025, D_CD.83.NDK, D_CD.83.ELI, F1_FR.96.MP411, F1_F1.93.FIN9363, F2_CM.95MP255, F2_CM.95MP257, G_SE.93.SE6165, G_BE.96.DRCBL, AE_TH.90.CM240, AE_CF.90.CF402, AG_IBNG, AG_FR.91.DJ263. An outgroup CPZ_CD.CPZANT was used.

also confirm in our study that group N is still a rare infection—only 1 (0.1%) group N infection in a total of 1469 HIV-1 samples (Ayouba et al., 2000).

Although the group-specific V3-loop peptide ELISA has limitations, it allows rapid screening of large numbers of samples to differentiate between HIV-1 groups. In addition, the technique is easy and can be easily implemented in developing countries where different HIV-1 groups co-circulate. As already previously described, the technique lacks sensitivity and cross-reactions between M and O are observed (Simon et al., 2001). Around 10% of our samples could not be typed; this is in concordance with results reported by Simon and colleagues on serum panels from Cameroon and Gabon (Simon et al., 2001). Lack of reaction with the V3 peptides may be due to low titers of V3 antibodies, suboptimal consensus sequences for the group M, O, or N peptides, or infection with divergent HIV strains. To test this latter possibility, we sequenced 17 untypable samples which represented 11.1% of the total untypable samples; all were group M strains without any particular V3-loop sequence. We also observed that a relatively significant number of the HIV-positive samples, 63 (4.3%), had simultaneously antibodies against group O and group M peptides. On a reference panel of 72 group M and 28 group O sera, Simon et al. (2001) showed that 2.8% of group M samples cross-reacted with the group O peptide and 29% of group O samples cross-reacted with group M peptide, but all groups reacted with the correct peptide and cross-reactions represent optical densities that are 50% lower. Our prevalences of dual O/M seropositivity correspond to the 5.7% documented by Simon and colleagues on a serum panel from Cameroon (Simon et al., 2001). We checked by PCR and sequencing the presence or not of group M and O viruses on a subset of 49 dual-seropositive

Table 3
Number and/or percentage of SI and NSI strains, according to the CD4 counts and for the different *env* (V3–V5) subtypes

| *env* subtype | <200 CD4 cells/mm$^3$ | | 200–500 CD4 cells/mm$^3$ | | >500 CD4 cells/mm$^3$ | | *n* tested |
|---|---|---|---|---|---|---|---|
| | SI | NSI | SI | NSI | SI | NSI | |
| A | 0 | 5 | 6 | 9 | 0 | 3 | 23 |
| C | 0 | 1 | 0 | 0 | 0 | 0 | 1 |
| D | 2 | 0 | 1 | 4 | 0 | 1 | 8 |
| F | 4 | 0 | 4 | 2 | 0 | 2 | 12 |
| G | 1 | 1 | 0 | 2 | 0 | 2 | 6 |
| H | 2 | 0 | 0 | 1 | 0 | 0 | 3 |
| K | 0 | 1 | 0 | 0 | 0 | 0 | 1 |
| CRF02 | 24 (55.8%) | 19 | 9 (20.0%) | 36 | 3 (20.0%) | 12 | 103 |
| CRF06 | 0 | 2 | 1 | 1 | 0 | 0 | 4 |
| CRF11 | 2 | 0 | 1 | 3 | 0 | 1 | 7 |
| Total | 35 (54.7%) | 29 | 22 (27.5%) | 58 | 3 (12.5%) | 21 | 168 |

samples, and documented the presence of at least two viruses in six samples. Moreover, in one sample reactive with only the group O peptide, we also documented dual infection. Different combinations of dual infections were documented; dual infection with groups O and M, dual infection with group O or M virus and an intergroup recombinant virus, with group M in the 5′ end and group O in the 3′ end, or vice versa. This means that at least in 7 (0.5%) of the 1469 HIV-1 infected patients, dual infection occurred, with the possible presence of an intergroup O/M recombinant in three patients. The minimal estimate of a 0.5% prevalence of dual infections is thus very close to the 1.0% of group O infections. Therefore, it will be important to monitor also for dual group M and O infection in Cameroon because recombination between strains belonging to distant lineages may contribute substantially to the emergence of new HIV-1 variants.

Our study also shows that group-specific PCR in two regions of the genome is an easy and specific tool with which to detect dual infections. The primers that we used in our study were validated with high sensitivities and specificities on viral isolates, whereas in our study the sensitivity could be lower because we started on primary material (plasma and/or PBMCs). The suboptimal storage conditions (−20°C for long periods) and lower viral loads in primary material can significantly decrease the sensitivity of the PCRs. However, the primers used were specific, since all fragments amplified with group-specific primers were confirmed by sequencing (data not shown, only for the dual-reactive samples in Fig. 3). This means that the numbers of dual infections (6/49) are minimal estimates and could probably be even higher. These intergroup recombinations could have important implications for diagnosis of HIV-1 infections by serological and molecular tests, and for treatment since differences among susceptibilities to nonnucleoside reverse transcriptase inhibitors (NNRTIs) exist between group O and group M (Descamps et al., 1997; Loussert-Ajaka et al., 1994). Actually, the low prevalence of group O infections does not yet compromise the use of nevirapine as prophylaxis for mother-to-child transmission of HIV, but our observations and other reports on dual infections suggest that intergroup O/M recombinants should also be monitored (Peeters et al., 1999a; Takehisa et al., 1997, 1999). Continuous monitoring of the different HIV-1 groups in Cameroon is also necessary to document the stabilization or decline of group O infection and the extent of dual infections and possible intergroup recombinants, and to find out whether group N is an emerging or declining HIV infection. To accurately identify group M and group O infection, the sensitivity of the V3-peptide assay has to be improved. The relatively low rate of confirmed dual infections, 12.2%, along dual-M/O-seropositive samples can be related to false-positive reactions, with one peptide, but also to the fact that one of the two viruses became predominant during

Table 4
Performance of V3-based phenotype prediction methods in non-B subtypes[a]

| Rule (prediction) | | NSI ($n$ = 108) | SI ($n$ = 60) | Sensitivity | Specificity |
|---|---|---|---|---|---|
| Positively charged amino acid at 11 or 25 | nsi | 103 | 40 | 33.3% | 95.4% |
| | si | 5 | 20 | | |
| Global charge | nsi | 66 | 15 | 75% | 61.1% |
| | si | 42 | 45 | | |

[a] Uppercase and lowercase letters denote experimentally determined phenotypes (NSI or SI) and predicted phenotypes (nsi or si), respectively. Sensitivity, fraction of correctly identified SI sequences; specificity, fraction of correctly identified NSI sequence (Resch et al., 2001).

HIV infection with viral loads under the detection limit, resulting in a negative PCR for the other infecting strain, but with persistence of specific antibodies. In addition, dual M/O infection was confirmed in one sample that reacted only with the group O peptide. This can probably be explained by the limit of the V3-peptide ELISA in which 10% of the samples remain untypable and thus not all group M infections are detected.

Based on partial *env* sequences, we demonstrated, in a random sample ($n = 295$), a high genetic diversity among HIV-1 group M strains, with at least eight subtypes and five CRFs that co-circulate. CRF02_AG is the predominant variant, representing more than 60% of the total HIV-1 infections. Within *env* subtype A sequences, we identified a separate subcluster, which we identified as A* for clarity in the text and figures, and the same strains clustered in *gag* with the CRF01_AE prototype strains. These strains clustered with the previously described CM53122 strain from Cameroon which is a mosaic virus between CRF01_AE and nonrecombinant subtype A (Carr et al., 2001). Heyndrickx et al. (2000) also identified three viruses as CRF01_AE in *gag* and A in *env*. It is thus possible that all these strains have a similar mosaic genome structure and represent a new CRF. Significant conclusions can be drawn only after sequencing two additional full-length genomes from epidemiologically unlinked individuals. In our study, 10% of the samples had discordant subtype/CRF designations between *gag* and *env*, and in the majority of the samples, CRF02_AG was involved in the possible recombination event, thus leading to more complex viruses.

In the present study, we evaluated the presence of SI and NSI variants in patients infected mainly with the CRF02_AG variant which predominates in West and West Central Africa. After stratification for CD4 counts, we observed similarly as for subtype B-infected patients (Fenyo et al., 1988; Karlsson et al., 1994; Koot et al., 1992, 1993; Tersmette et al., 1988, 1989) that the proportion of SI strains increases when disease is more advanced and CD4 counts are lower. We can therefore conclude that the biological characteristics of the CRF02_AG strains are similar as those seen in subtype B-infected patients in Europe and the United States. It is, however, important to note that the two predominant HIV-1 variants in Africa, CRF02_AG and subtype C, have different biological characteristics. Multiple reports have shown that CXCR4 usage as well as MT-2 cell tropism was rare among subtype C samples (Bjorndal et al., 1999; Peeters et al., 1999b; Tscherning et al., 1998).

For subtype B, positively charged amino acids are found at positions 11 and/or 25 in the V3 loop of SI isolates, whereas in NSI isolates both residues were either uncharged or negatively charged (De Jong et al., 1992; Fouchier et al., 1992). However, in our study, 95.4% of the NSI strains in vitro were predicted NSI, but only 33.3% of the SI strains were predicted SI, suggesting that the in vitro detection of SI variants cannot be replaced by a genetic method for CRF02. Other reports also observed a lack of correlation between the V3-loop amino acid sequence and the in vitro SI capacity of some HIV-1 isolates, and this lack of correlation is observed among different genetic subtypes including subtype B (Holm-Hansen et al., 1995; Peeters et al., 1999b; Sabri et al., 1996). Similarly, despite the fact that the global charge of the V3 loop was significantly lower for NSI strains, this cannot be used as an individual marker to predict whether strains are SI or NSI.

Our study also shows that co-infection and recombination between divergent HIV-1 strains is thus not as rare as initially thought. To develop an efficient vaccine, it remains to be determined when superinfection can occur during the course of HIV infection, and to what extent humoral and cellular antibody responses are efficient against divergent strains. Differences between transmissibility and pathogenicity have been well documented for HIV-1 and HIV-2, with HIV-2 being less transmissible and less pathogenic than HIV-1 (De Cock et al., 1993; Kanki et al., 1994; Marlink et al., 1994). Whether similar differences exist among the HIV-1 groups and/or subtypes has to be studied further. Recently, a study in Uganda documented on more than 1000 patients that compared with subtype A, subtype D is associated with a faster progression to disease (Kaleebu et al., 2002). We recently showed that patients infected with the CRF02_AG variant have a progression to disease similar to that of patients infected with non-CRF02_AG strains (Laurent et al., 2002). Associations between modes of transmission and subtype are probably due more to historical chance coupled with behavioral factors, but biological factors related to the host and the virus could also play a role (Hu et al., 1999; Kunanusont et al., 1995; van Harmelen et al., 1997). NSI variants seem to be more readily transmitted than SI variants (Cornelissen et al., 1995; Zhu et al., 1993). Therefore, it is important to follow-up whether the spread of certain variants, for example CRF02_AG and subtype C, in Africa could be associated with their biological characteristics.

## Materials and methods

### *Patients and serology*

All HIV-1-positive samples from patients attending PRESICA, a health structure providing counselling, voluntary testing, and medical care for HIV-infected patients based at the military hospital in Yaounde, were systematically tested to differentiate between HIV-1 group O, M, and N and HIV-2 infection, over a 6-year period (1996–2001). The military hospital in Yaounde, the capital city, is one of three major referral centers for patients with suspected HIV-related disease. Most patients are referred directly to PRESICA from other hospitals or primary health care facilities for suspected HIV-related diseases or for HIV screening. Since January 1997, a cohort study has been initiated to study the impact of the different genetic sub-

types/CRFs on disease progression. Patients identified as HIV infected who were more than 14 years old, and with a Karnofsky index greater than 70, were asked to participate in this cohort study. The patients enrolled in the cohort were asked to attend medical examinations quarterly or more often if necessary. Immunological status was recorded at baseline and then every 6 months; the genetic subtype was identified and when possible viral cultures were attempted. All patients received pre- and posttest counselling, and HIV testing was done after written informed consent was received from the patient. All clinical and laboratory tests were free of charge at the initial visit, as were medical visits and laboratory tests at follow-up for those participating in the cohort. Free medical care, including prophylaxis and treatment of opportunistic infections, was offered to each patient with a positive HIV serology.

Screening for HIV-1 antibodies was performed by ELISA (Murex HIV-1.2.0, Abbott, Rungis, France) followed by confirmation on a Line immunoassay, INNOLIA HIV-1/HIV-2 (Innogenetics, Belgium). CD4+ cell counts were determined by flow cytometry (FACSCount, Becton Dickinson, Mountain View, CA, USA) in freshly collected whole blood at baseline and then every 6 months. Since 2001, antiretroviral drugs (ARVs) have been offered to all patients with medical criteria, based on their clinical evolution and CD4 counts as defined in a consensus report on the place of ARVs in Africa (IAS, 1999).

To differentiate between HIV-1 group O, M, and N and HIV-2 infection, sera were tested with an in-house indirect ELISA based on synthetic V3-loop peptides of HIV-1-M, -N, and -O, and HIV-2, as previously described (Simon et al., 2001). Briefly, wells of microtiter plates were coated with 0.25 $\mu$g of each single peptide per well in 0.05 M bicarbonate buffer, pH 9.6, by incubation at 37°C for 20 h. After washing with phosphate-buffered saline (PBS) containing 0.5% Tween 20, unoccupied sites were blocked with PBS containing 5% fetal calf serum, for 2 h at 37°C. Sera were diluted 1/100 in an hypertonic PBS solution (0.01 M sodium phosphate buffer, pH 7.4, containing 0.75 M NaCl, 10% fetal calf serum, and 0.5% Tween 20). After incubation for 30 min at room temperature, plates were washed and incubated with peroxidase-conjugated goat anti-human IgG for 30 min at room temperature. After washing, the reaction was revealed with hydrogen peroxide–*o*-phenylendiamine for 15 min at room temperature in the dark. Color development was stopped by adding 2 N $H_2SO_4$, and OD was read at 492 nm.

### *Genetic subtyping in the gag and env regions*

Blood was collected into EDTA tubes, and plasma and cells were separated and stored at −20°C. DNA was extracted from the primary or cultured peripheral blood mononuclear cells (PBMCs) using the Qiagen DNA isolation kit (Qiagen SA, Courtaboeuf, France), according to the manufacturer's instructions.

Seven hundred-base pair fragments, corresponding to the p24 region from the *gag* gene and the V3–V5 *env* region, were amplified as previously described by a nested PCR (Toure-Kane et al., 2000). The amplified fragments were purified using the QIAquick gel extraction kit (Qiagen) and were directly sequenced with the Abiprism BigDye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA polymerase (FS; Perkin–Elmer, Roissy, France) on an automated sequencer (373A stretch; Applied Biosystems).

### *Identification of HIV-1 group O and M sequences in dual group O and M seropositive samples*

Plasma or primary PBMCs from dual-seropositive samples were tested by RT-PCR or PCR respectively, with group M- and group O-specific primers in *env* (C2V3 and gp41) and *pol* (partial integrase), to confirm the simultaneous presence of both viruses. The group M C2V3 fragment was amplified with a nested PCR using previously described group-M specific primers ED5/ED12 as the outers, and ED31/ED33 as the inners under previously described PCR conditions (Delwart et al., 1993). The HIV-1 group O C2V3 region was amplified as previously described in a nested PCR with the following primers as outers,

V70.1: 5′-TTGTACACATGGCATTAGGCCAACAG-TAAGT-3′
V70.5: 5′-GTTCTCCATATATCTTTCATATCTCCC-CCTA-3′

and as inners,

V70.2: 5′-TGAATTCCTAATATTGAATGGGACAC-TCTCT-3′
V70.4: 5′-TGGATCCTACAATAAAAGAATTCTCC-ATGACA-3′

(Peeters et al., 1997). Although the sensitivity of the C2V3 group O primers was high (first and second round specific to group O), an additional PCR was done in the gp41 region on the samples remaining negative with group O-specific primers in the C2V3 region. A nested PCR was done with universal group M and O primers as outers,

gp41.1: 5′-GGGTTCTTGGGAGCAGCAGGAAGCA-CTATGGGCG-3′
gp41.4: 5′-TCTGAAACGACAGAGGTGAGTATCC-CTGCCTAA-3′

and with group O-specific primers as inners,

gp41.6: 5′-TGGATCCCACAGTGTACTGAAGGGT-ATAGTGCA-3′
gp41.7: 5′-CATTTAGTTATGTCAAGCCAATTCCA-AA-3′

under previously described conditions (Bibollet-Ruche et al., 1998). The amplified *env* fragments were purified, directly sequenced, and analyzed by phylogeny.

To discriminate between HIV-1 groups O and M in the *pol* gene, the following primers were used in a nested PCR. A universal first round was done with 4235 (5′-CCCTACAATCCCCAAAGTCAAGG-3′) and 4538 (5′-TACTGCCCCTTCACCTTTCCA-3′) primers and a second round with either a group-M specific primer (4241M: 5′-TAGAATCTATGAATAAAGAATTAAAGAA-3′) and a universal primer (4481: 5′-GCTGTCCCTGTAATAAACCCG-3′) or group O-specific primers (4241-O: 5′-TAGAAGCCATGAATAAGGAATTAAAATC-3′; 4391-O: 5′-TTTGTAATTCTGTTGTTTGTATTTGTGA-3′) (Heyndrickx et al., 1998; Janssens et al., 1995).

*Phylogenetic analysis*

Nucleotide sequences were aligned using CLUSTAL W with minor manual adjustments, bearing in mind the protein sequences (Thompson et al., 1994). Regions that could not be aligned unambigously, due to length or sequence variability, were omitted from the analysis. Phylogenetic trees using the neighbor-joining method and reliability of the branching orders using the bootstrap approach were implemented with CLUSTAL W (Saitou and Nei, 1987). Genetic distances were calculated with Kimura's (1981) two-parameter method (ratio $t/T = 2.0$). The newly determined HIV-1 *env* and *gag* sequences were aligned with known HIV-1 sequences representing the different genetic subtypes and sub-subtypes and reference strains from the CRFs documented in West and West Central Africa (CRF01_AE, CRF02_AG, CRF06_cpx, CRF11_cpx and CRF13_cpx) (Peeters, 2000; Wilbe et al., 2002). To clearly identify whether a sequence belonged to a subgroup corresponding to a CRF within a certain subtype or not, phylogenetic analysis was done for each sequence individually. Then, different trees were constructed for each group of new sequences that were thought to cluster together and, finally, a general tree was obtained to visualize all the results. The clustering of each new sequence was compared and should be concordant between all trees. In the same way, for the dual-reactive samples, phylogeny analyses were performed on group M and O sequences against reference sequences in the *env* gene and in the *pol* gene to confirm the amplification with group-specific primers (phylogeny on a 220-bp fragment is not significant but is informative in checking the HIV-1 group). The reference strains used are indicated in the legends of the phylogenetic trees.

*Determination of syncytium-inducing capacity*

PBMCs from the HIV-positive patients were co-cultivated with phytohemaglutinin (PHA)-stimulated lymphocytes from a HIV-negative human donor in RPMI-1640 medium (Biowhittaker, Verviers, Belgium) supplemented with 15% heat-inactivated fetal calf serum (Gibco, Paisley, Scotland), 0.03% L-glutamine (Gibco), 2 $\mu$g of Polybrene (Murex, Dartford, England) per milliliter, antibiotics, and 20 U of recombinant interleukin-2 (Boehringer, Mannheim, Germany) per milliliter. The release of viral particles in the culture supernatants was examined with a HIV p24 antigen-capturing test (Innogenetics, Gent, Belgium).

Syncytium formation was determined with the MT-2 assay essentially as described by Koot et al. (1992). HIV-1 strains obtained from the initial culture were propagated by short-term passage (7–10 days), and then 1 million infected PBMCs were co-cultivated with 2 million MT-2 cells at a concentration of 500,000 cells/ml. HIV-1 cultures were considered to exhibit syncytia if one multinucleated giant cell per field of the light microscope was observed. We studied the prediction of the phenotype based on the sequence of the V3 loop, i.e., we examined whether basic or positively charged amino acids (K, R, or H) at positions 11 and/or 25 in the V3 loop were present, and we calculated the global charge of the V3 loop (De Jong et al., 1992; Fouchier et al., 1992).

*Statistics*

Data were analyzed using Stata 7.0 software (Stata Corporation, College Station, TX, USA). For prevalences of HIV-1 group M, N, and O and HIV-2 infections, 95% confidence intervals (CIS) were computed using the binomial exact method. The trends over time were assessed by use of the $\chi^2$ test for trends. The global charge of the V3 loop in SI and NSI groups was described by the mean and the standard deviation, after checking the distribution normality with the Shapiro–Francia $W$ test. Comparisons between SI and NSI groups were then based on the two-sample $t$ test.

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