Interpatient genetic variability of HIV-1 group O

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Objective: To analyse the genetic and phylogenetic characteristics of HIV-1 group O viruses.

Materials and methods: The *env* gene, encoding the gp160 glycoprotein, and a partial p24-encoding *gag* gene fragment of a Cameroonian (CA9) and a Gabonese (VI686) HIV-1 group O virus, isolated from cultured peripheral blood mononuclear cells of symptomatic patients, were sequenced, aligned with other representatives of group O for which the same region has been documented, and genetically and phylogenetically analysed.

Results: Phylogenetic analysis of the *env* gene (gp160) revealed that CA9, VI686, ANT70, and four Ha strains formed a separate cluster, which was supported by 100% of all bootstrap trees. In addition, these seven isolates were part of the same clade in the p24 phylogeny. VAU and MVP5180 may represent two other subtypes.

Conclusion: We have characterized two group O viruses, originating from Cameroon and Gabon, which show a close evolutionary relationship to ANT70 and four Ha strains based on the entire *env* gene, suggestive of a first group O subgroup, tentatively named the HIV-1 group O *env* ANT70 clade or subtype.

AIDS 1999, **13**:41–48

Keywords: HIV-1 group O, variability, subtype, *env* gp160, *gag* p24

Introduction

Phylogenetic analysis of HIV-1 strains isolated from individuals from different geographical regions has resulted in the assumption of a two-star phylogeny, whereby two different groups are distinguished, group M (major) and group O (outlier) [1]. Our limited knowledge of the epidemiology of infections due to group O viruses is based on serology [2–4]. Besides Cameroon and Gabon, evidence for group O infections has been reported from other African countries including Equatorial Guinea [5], Bénin [6], Senegal [4], Togo [4], Niger [4], Chad [4], Nigeria [4,7] and Kenya [4,8]. Outside Africa, group O viruses have been documented in Europe (Belgium [9], France [10], Germany [11], Spain [12], and Norway [13]) and the United States [14]. Dual infections with group O and group M viruses have been described in Bénin [6], Kenya [8] and Cameroon [15].

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Sponsorship: This work was supported by grant number 3-3301-96 and G.0134.97 of the Fonds voor Wetenschappelijk Onderzoek, Brussels, Belgium, EC grant numbers EC18-CT96-0110 and IC18-CT97-02476; and The Flanders Interuniversity Institute for Biotechnology, VIB, Zwijnaarde, Belgium.

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Date of receipt: 23 April 1998; revised: 26 August 1998; accepted: 7 October 1998.

Further characterization of aberrant strains is required because some group O viruses are missed by certain HIV antibody tests [16,17]. In addition, whereas about 34% of sera obtained from persons infected by group M viruses neutralize certain group O variants, sera from group O-infected individuals consistently fail to neutralize group M viruses [18,19]. Furthermore, some group O viruses are highly resistant to non-nucleoside reverse transcriptase inhibitors [20,21]. Although cyclophilin A inhibits HIV-1 group M viral production, it does not affect HIV-1 group O viral replication [22].

The phylogenetic classification of group O strains into subtypes has not yet been addressed, mainly because the group O strains for which C2V3 *env* and p24 *gag* sequence data were obtained do not appear to cluster into subtypes as observed for HIV-1 group M. There is only a poor association between phylogenetic classification based on C2V3 *env* compared with p24 *gag* [10]. In this study we compared the phylogenetic relationship of HIV-1 group O strains CA9, VI686 and previously documented group O viruses, on the basis of the entire gp160-encoding *env* gene and partial p24-encoding *gag* fragment.

Materials and methods

Patients

CA9 and VI686 were isolated from cocultured peripheral blood mononuclear cells from a Cameroonian patient and a Gabonese patient, respectively, both of whom were symptomatic. Sera obtained from these patients were initially reactive in a solid-phase group O V3-loop enzyme-linked immunosorbent assay (ELISA) [23]. Group O infection was later confirmed by PCR, sequencing and phylogenetic analysis of part of the *pol* gene [24]. A 900 base-pair *env* fragment of VI686 encoding V3, V4, V5, and the start of gp41 was previously reported in a study on epidemiological and molecular characteristics of HIV infection in Gabon [25]. For HIV-1 CA9, CC-chemokine receptor (CCR)-5 was identified as the main coreceptor for HIV-1 entry into CD4+ cells of the immune system [26].

Viral phenotypic analysis

The GHOST cell line used was derived from HOS (human osteosarcoma) cells. HOS cells were transfected with genes coding for human CD4, one of the eight chemokine receptors (CCR-1, CCR-2b, CCR-3, CCR-4, CCR-5, CXCR-4, BOB, and BONZO), which function as HIV-1 coreceptors, and an indicator gene under the control of an HIV-2 promoter, inducible by *tat*, which permitted evaluation and quantification of HIV-1 infection by flow cytometry [27]. Briefly, cells were seeded in 24-well plates at a concentration of 6×10^4 cells/well. On the following day, cells were infected with undiluted virus stocks in combination with diethylaminoethyl–dextran to a final concentration of 8 μg/ml. After overnight incubation, the virus inoculum was removed and the cells were washed with phosphate-buffered saline (PBS). After 4–5 days, the cells were washed again with PBS, resuspended in 1 mmol/l EDTA and fixed in formaldehyde at a final concentration of 2%. The cells were then analysed with FACS analysis. The live cells were gated on the basis of forward and side scatter. The number of infected cells was determined using the scattergram of fluorescence versus forward scatter after setting the gates with uninfected cells. More than 99 cells out of every 15 000 cells had to be present for a virus to be considered positive for infectivity in each of the GHOST cell lines tested [27].

Virus isolation, preparation of DNA, PCR, cloning and sequencing

Virus isolation and preparation of DNA template for PCR were as described previously [28]. The *env* gene of CA9 and VI686 was amplified by PCR using PCR primers designed based on ANT70 and MVP5180 sequence information. Four fragments were amplified. These overlapped and together gave the full envelope coding sequence. For VI686 and CA9, the sequences were obtained from the following PCR amplified fragments, starting from the gp160 signal sequence (nucleotide positions refer to the reported sequences of VI686 and CA9): VI686, 1–1164, 818–2043, 1951–2274, 2062–2640; CA9, 1–999, 815–2034, 1942–2265, 2053–2631. Primers for amplification of the p24-encoding *gag* fragment were GAG5CAM and GAG3CAM [10]. All PCR fragments were ligated in pUC18 (Pharmacia Biotech Benelux, Roosendaal, The Netherlands) or pGEM-T (Promega Benelux, Leiden, The Netherlands). One clone was sequenced for each *env* and *gag* fragment. Purification of plasmid DNA was performed using the Qiagen plasmid kit (Westburg, Leusden, The Netherlands) prior to sequencing using fluorescein-labelled primers. Electrophoresis and data collection were performed using an automatic laser fluorescence (ALF) sequencer (Pharmacia Biotech Benelux).

Phylogenetic analysis

The entire HIV-1 *env* sequences of CA9 and VI686 were aligned with 28 known sequences of HIV-1 isolates of diverse geographical origin on the basis of primary nucleotide structure. All group M isolates that were listed as subtype representatives in the Los Alamos database [29] and that had been characterized on the basis of full-length genome sequences [30] were included in the analysis. In addition, two subtype G sequences reported by Carr *et al*. [31], and also analysed on the basis of their full-length genome sequences, were used. All previously reported complete group O

gp160 sequences were included (i.e., ANT70, MVP5180, VAU, 193Ha, 267Ha, 341Ha, 655Ha) [32,33]. For phylogenetic analysis of the *gag* p24 coding region, the same isolates from group M were used, in addition to the following group O strains: ANT70, MVP5180 [32], BCF01, BCF02, BCF03, BCF06, BCF07, BCF08, BCF11 [10], 193Ha, 267Ha, 341Ha, 655Ha, ABT063, ABT124, ABT325, ABT2156 [34] and DUR (unpublished, European Molecular Biology Laboratory (EMBL) accession number X84329).

The sequences were aligned automatically using CLUSTAL W [35], and the resulting alignment was refined manually with the dedicated comparative sequence editor (DCSE) [36]. The DCSE software is a multiple alignment tool that can be used for manually editing amino-acid and nucleotide alignments. Regions that could not be aligned unambiguously due to excessive length or sequence variation were omitted from the analysis. The software package TREECON [37] was used for distance calculations (Jukes and Cantor), tree construction (neighbour-joining) and bootstrap analysis. The nucleotide sequence data were deposited in the EMBL, GenBank and DNA Data Bank of Japan (DDBJ) nucleotide sequence databases under the following accession numbers: X96522 (CA9 *env),* X96524 (CA9 *gag*), X96526 (VI686 *env),* X96529 (VI686 *gag*).

Results

Viral phenotypic analysis

The coreceptor usage of CA9 and VI686 was examined on GHOST-3 cells, expressing CCR-1, CCR-2b, CCR-3, CCR-4, CCR-5, CXCR-4, BOB, or BONZO. Virus infection could be measured on GHOST–CCR-5 cells for both CA9 and VI686. No virus infection was measured on the cells expressing the other coreceptors. These results indicated that both viruses had a non-syncytium-inducing (NSI) phenotype.

Phylogenetic analysis

The complete gp160 glycoprotein encoded by the *env* gene and part of the p24 protein encoded by the *gag* gene of HIV-1 isolates VI686 and CA9 were sequenced. These sequences were aligned with other representatives of group O and M isolates from which the same regions had previously been documented. Phylogenetic trees were generated on the basis of the

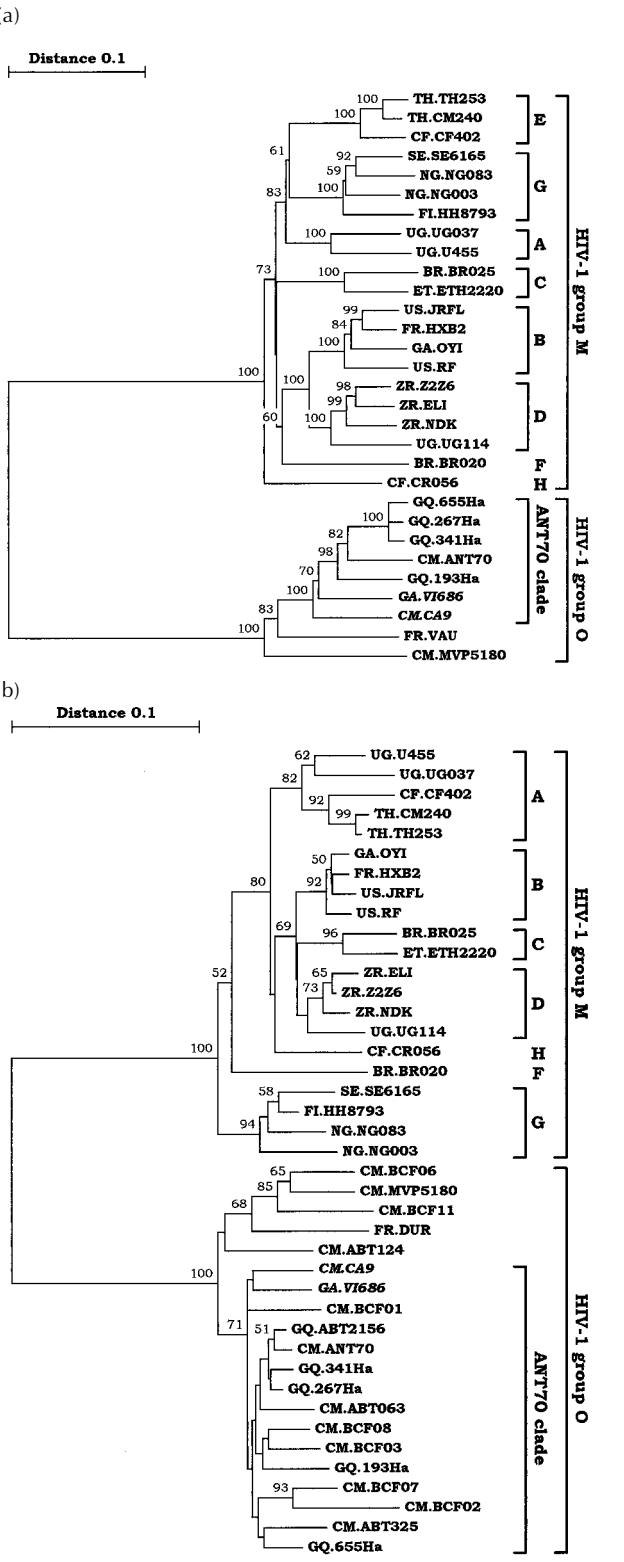

Fig. 1. Phylogenetic trees of HIV-1 nucleotide sequences constructed using the neighbour-joining method, and using the Jukes and Cantor correction for estimating evolutionary distances from dissimilarities. Insertions and deletions were not taken into account. Bootstrap values from a 2000 replicate analysis are given in percentages at the internodes if they exceed the 50% level. Sequences that were determined in this study are shown in italics. The distance between two sequences is obtained by summing the lengths of the connecting horizontal branches, using the scale at the top. The root of the trees was artificially placed between group M and O sequences. Only well-aligned positions were used: (a) 2311 positions of an alignment containing complete gp160 *env* sequences; (b) 408 positions of a partial p24 *gag* sequence alignment.

Table 1. Overview of the evolutionary distances of group M and O isolates for different datasets*.

	gp160 *env*	p24 *gag*
Highest distances in groups		
Within group M†	19.9 (BR025–HH8793)	16.0 (BR020–CF402)
Within group O	19.4 (VAU–MVP5180)	15.8 (BCF02–BCF06)
Highest intrasubtype distances		
Within group M subtypes	10.6 (UG114–ELI)	8.6 (CF402–UG037)
Within ANT70 clade	13.1 (655Ha–CA9)	12.0 (193Ha–BCF02)
Lowest intersubtype distances		
Between group M subtypes	11.4 (Z2Z6–HXB2)	4.4 (Z2Z6–JRFL)
		4.4 (Z2Z6–HXB2)
		4.4 (ELI–JRFL)
		4.4 (ELI–HXB2)
ANT70 clade and other O strains	15.5 (VI686–VAU)	8.8 (ABT124–BCF08)
		8.8 (ABT124–ABT2156)

*Distances between the isolates (in parentheses) are given as percentages, and were calculated as described in Fig. 1. For the calculation of these distances, all sequences that were included in the trees of Fig. 1 were taken into account. †For each of the two datasets, both isolates belong to a different subtype.

complete *env* gp160 (Fig. 1a) and the partial *gag* p24 (Fig. 1b) coding regions, for which sequence data from more isolates were available. Based on these datasets, inter- and intrasubtype evolutionary distances from group O and M viruses were compared (Table 1).

In the tree based on the complete *env* sequence information (Fig. 1a), the HIV-1 group M subtypes of which more than one representative was included, were all highly supported by bootstrap values of 100%. Amongst the HIV-1 group O isolates, a cluster consist-

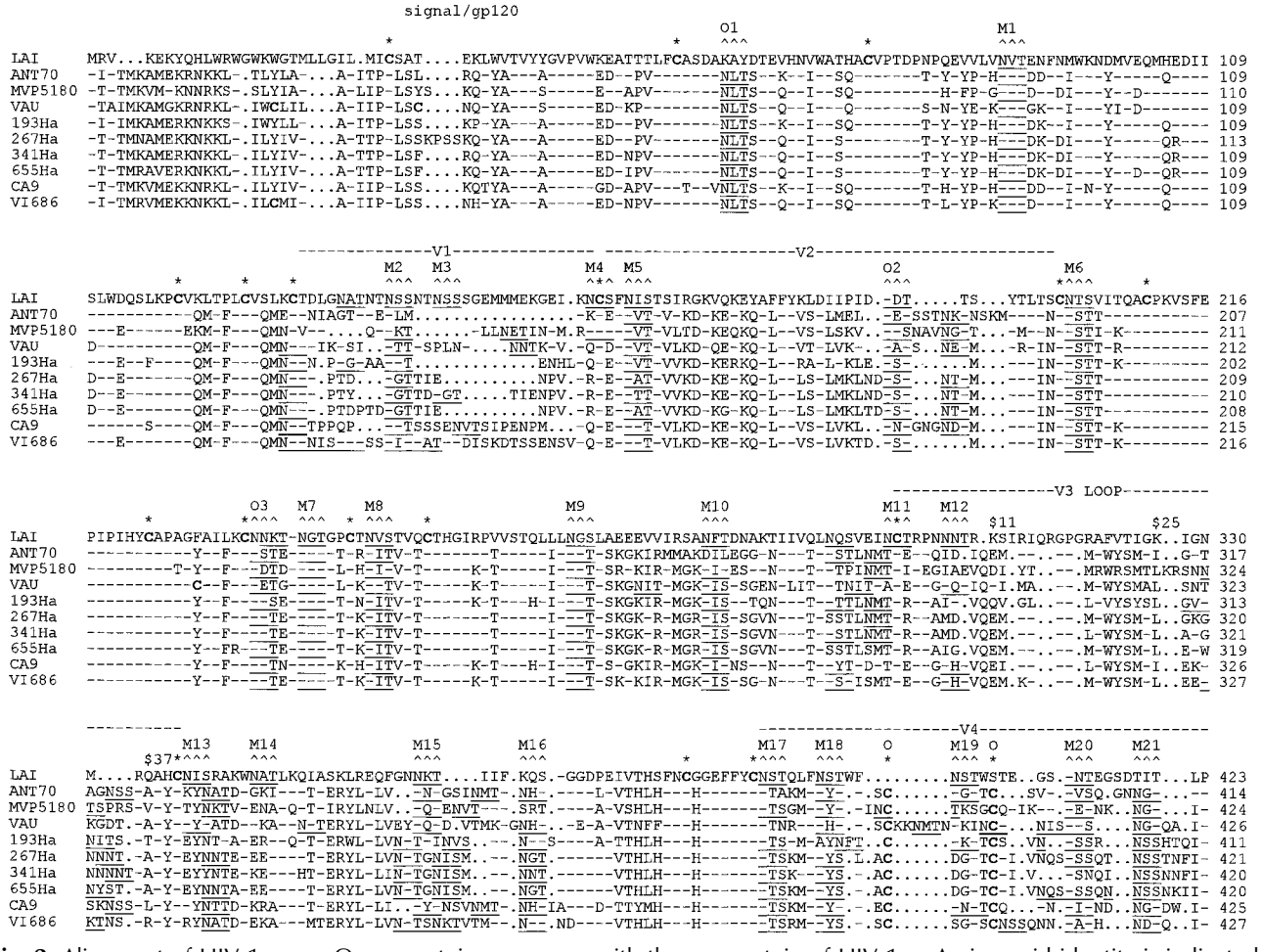

Fig. 2. Alignment of HIV-1 group O *env* protein sequences with the *env* protein of HIV-1_{LAI}. Amino-acid identity is indicated by dashes; insertions and deletions are indicated by dots. The potential N-linked glycosylation sites are underlined; those highly conserved among group M or group O isolates are indicated with M or O above carets. *Highly conserved cysteine residues. Two additional cysteine residues, conserved in group O isolates, are indicated with O above an asterisk. Amino-acid positions 11, 25 and 37 of the V3 loop, which are determinants of the syncytium-inducing phenotype, are indicated by $.

ing of isolates ANT70, CA9, VI686 and four Ha sequences, which will be referred to as the ANT70 clade, was also supported by 100% of the bootstrap trees.

In the tree generated using the partial *gag* p24 coding sequences (Fig. 1b), the bootstrap support for the different group M subtypes was much lower, and for subtype D it was below 50%. Within group O, the isolates from the ANT70 clade in Fig. 1a belonged to a cluster that contained additional sequences. This ANT70 clade was rather poorly supported by a bootstrap value of 71%. Within this clade, relationships were not generally supported by bootstrap analysis. The isolates BCF06 and BCF11 were grouped with MVP5180, supported by an 85% bootstrap value. The p24 coding sequence information of VAU was not available.

Amino-acid sequences analysis

The CA9 and VI686 gp160 sequences were aligned with the previously documented group O sequences (ANT70, MVP5180, VAU, 193Ha, 267Ha, 341Ha, 655Ha), and a representative of group M (LAI; Fig. 2). The *env* gene, including the signal peptide, encoded 876 (CA9) and 879 (VI686) amino acids.

The pattern of potential N-linked glycosylation (consensus motif, N-X-S/T) in the envelope was as follows. CA9 and VI686 had 34 and 36 potential N-linked glycosylation sites, respectively (Fig. 2). Twenty-eight sequons were common to most documented group M isolates [24] (indicated by M1 to M28; Fig. 2), of which 24 were also present in CA9 and VI686. Altogether, 12 out of 28 common potential N-linked glycosylation sites for group M isolates were also present in all currently documented group O isolates: M1, M5, M6, M7, M8, M9, M15, M18, M21, M23 in gp120 (of which M21 was absent in LAI), and M25, M28 in gp41. M2, M10, M13, M16 and M20 were present in all but one group O isolates. In addition, five potential N-linked glycosylation sites were present in group O isolates (indicated by O1 to O5 in Fig. 2); four were localized in gp120 (O1–O4, of which O4 was absent in VAU), and one in gp41 (O5). The pattern of cysteine residues distribution was as follows: 19 conserved cysteine residues in group M gp120, as well as two in gp41 (CA9 is missing the first one) were maintained for the group O isolates. Two additional cysteine residues were present in the gp120 V4 domain as well as in gp41 (VAU lacked the second residue) for group O specimens.

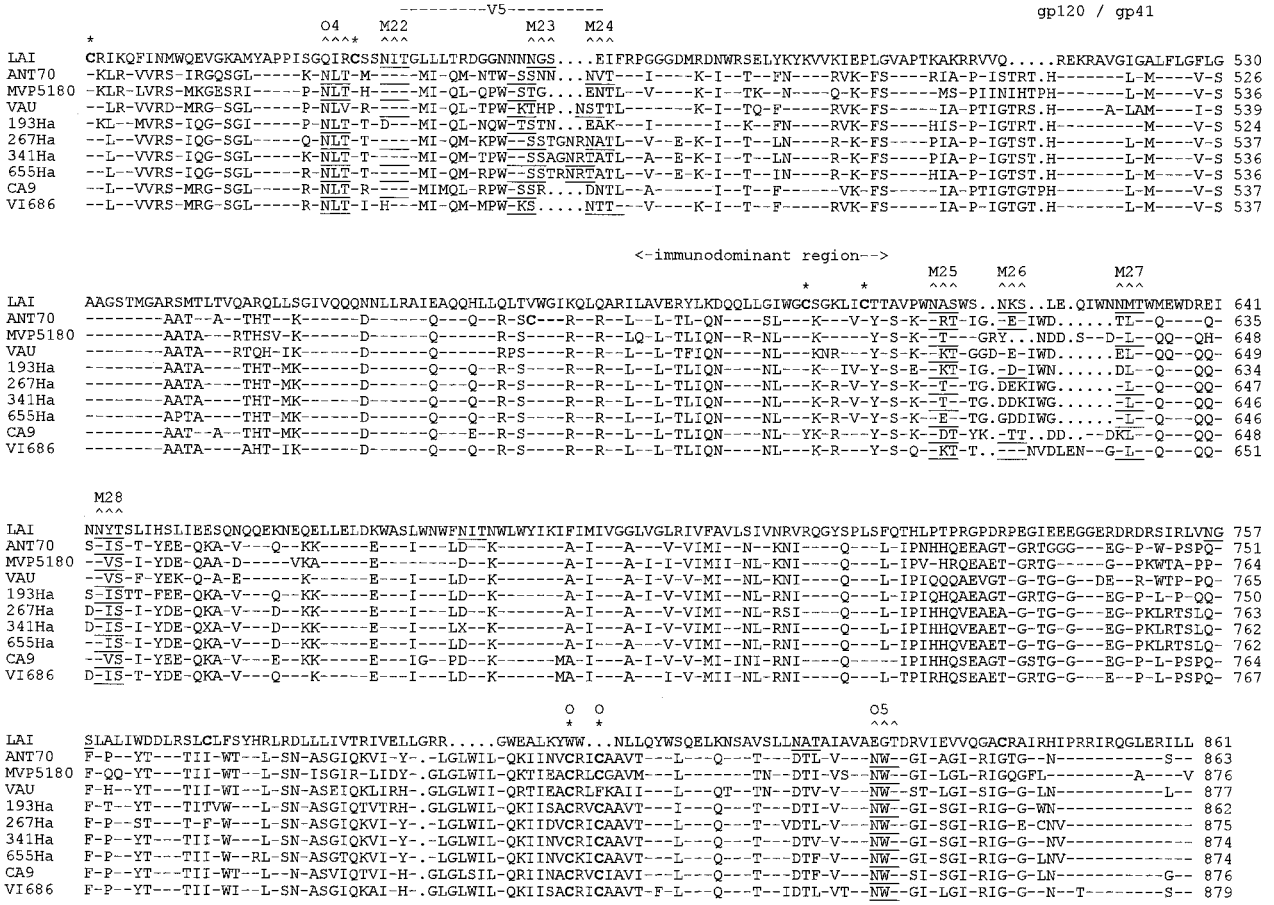

Fig. 2. (continued.)

The principal neutralizing domains or V3 loops of CA9 and VI686 were 37 and 36 amino acids long, respectively. The octamer in the tip of the V3 loop was RIGPLAWY and KIGPMAWY for CA9 and VI686, respectively. An overall low net charge and a positively charged amino acid (arginine) at position \$37 (Fig. 2) of the V3 loop of VI686 were indicative of an NSI/MT-2-tropic isolate [38].

Discussion

HIV-1 groups M and O are considered to have evolved from two centres of evolutionary HIV-1 radiation, each as a result of at least one zoonotic transmission. Phylogenetically, this is represented by a double star [39]. HIV-1 group M is currently subdivided into 10 distinct *env* subtypes, which are approximately equidistant to each other in terms of genetic distance. Comparisons of *gag* sequences leads to a similar classification [39,40]. For subtyping of HIV-1 group M strains, phylogenetic analysis of sequences encoding the *env* C2V3 region is considered adequate, based on a perfect agreement with subtype determinations on long *env* sequences [41]. In a previous search for HIV-1 group O subtype designations, phylogenetic analysis comparing HIV-1 *env* encoding C2V3 and *gag* encoding p24 only revealed an association between two isolates, BCF02 and BCF07 [10]. These observations are in agreement with our experience that the C2V3 region is inadequate for phylogenetic analysis of group O sequences (unpublished). This could be due to considerable length and sequence variation in C2V3, which makes the region too variable for reliable phylogenetic analyses. Moreover, due to this variability, only a few more than 200 alignment positions can be reliably aligned within group O, which is inadequate for constructing well-supported phylogenies.

Our phylogenetic analyses comparing the complete *env* genes, including CA9 and VI686, revealed a cluster of seven isolates (ANT70, CA9, VI686, and four Ha strains), which was supported in 100% of all bootstrap trees (Fig. 1a). This may be considered the first documented subtype in HIV-1 group O, tentatively named the HIV-1 group O *env* ANT70 clade. Alternatively, the ANT70 subtype could be limited to only ANT70 and the four Ha strains, excluding VI686 and CA9. However, this seems less likely at this time, because VI686 and CA9, belong to the ANT70 cluster in the p24 phylogeny as well. However, it should be noted that in the p24 phylogeny, the ANT70 cluster is only supported by a bootstrap value of 71%. However, since the support of group M subtype D in this tree is even less than 50%, this is no real argument against the existence of the ANT70 subtype. Judging from the gp160 phylogeny (Fig. 1a), VAU and MVP5180 may represent other subtypes, which in the case of MVP5180 could be supported by the p24 tree (Fig. 1b). In the latter tree, MVP5180 together with BCF06 and BCF11 might represent another subtype, or alternatively DUR might also be included. Of course, one should realize that the recognition of subtypes depends on which sequences are available. As with the group M subtypes, group O subtypes, if they really exist, will also have to be defined as more isolates are characterized.

Table 1 shows that the highest distance observed between two isolates of group M is nearly identical to the highest distance in group O. This indicates that the overall variation in both groups is very similar. On the other hand, the highest distance observed within the ANT70 clade is considerably higher than the highest distance within any group M subtype. Moreover, the lowest distance between two group M isolates of different subtypes is much smaller than the lowest distance observed between any ANT70 subtype strain and other group O viruses. These values might illustrate that the variation within group O subtypes is higher than that within group M clades, and that at least some group M subtypes are more related to one another than are group O clades. However, until more group O sequences are available, it is far too early to draw any firm conclusions for group O, and the higher values for group O compared with group M could merely be a reflection of the way subtypes are currently defined, certainly if one takes into account the possibility that genetic distances between two group M isolates can be as high as those between two group O viruses.

Compared with group M viruses, four additional group O potential N-glycosylation sites in gp120 (of which one is absent in VAU) and one in gp41 are conserved. In addition to 21 cysteine residues (of which one is absent in CA9) that are shared amongst HIV-1 group O and group M, group O viruses have four cysteine residues, two in the fourth variable domain of gp120 and two in gp41, of which one is absent in VAU. The number and positioning of N-glycosylation sites, as well as the number and location of cysteine residues, which are likely to be involved in intramolecular disulphide bridges, might have consequences in protein folding, and may thus result in an altered presentation of envelope epitopes, compared with HIV-1 group M [42,43]. This might in some cases result in an altered immune response, leading to escape of detection by currently available antibody assays, atypical reaction patterns in Western blot, and possibly the lack of neutralization of group M viruses by serum obtained from individuals infected by group O viruses.

As a consequence of the heterogeneity of HIV-1 group O relative to group M, antibodies induced by VI686 could not be detected in Wellcome HIV-1 and 2 ELISA (Wellcome Diagnostics, Dartford, Kent, UK)

and was indeterminate in HIV-1 Western blot. This is consistent with previous reports for group O viruses. Loussert-Ajaka *et al.* [16] reported HIV-1/2 seronegativity in ELISA systems using synthetic peptides as solid-phase antigens in individuals infected with group O viruses. The alignment of the immunodominant region in gp41, which is an important diagnostic epitope [44], results in a consensus sequence for group O, RLLALETLIQNQQLLNLWGCKGRLIC, which is shared by both CA9 and VI686 (except for the first cysteine residue in CA9), and which is markedly different from the group M immunodominant consensus peptide, RVLAVERYLKDQQLLGIWGCSGKLIC. From these consensus sequences it is clear that certain amino acids that are highly conserved in group M strains are not conserved in group O strains and *vice versa*. Strikingly, at position 21 of the immunodominant group O peptide, serine (S) is replaced by lysine (K). Previous findings, together with ours, highlight the effect of considerable sequence divergence of HIV-1 isolates on serodiagnosis. Although assays that lacked sensitivity for group O viruses have been withdrawn from the market, including Wellcome HIV-1 and 2, there is still a need to constantly evaluate newer versions of commercially available assays for their sensitivity to highly divergent viruses. Moreover, monitoring and characterizing divergent strains in each community, especially in endemic areas, should remain a cornerstone for HIV research in order to ensure transfusion safety.

The observed genetic diversity between HIV-1 group O and group M viruses also reflects antigenic diversity. Antibodies from VI498- (a follow-up sample of ANT70) and CA9-infected individuals have a reduced capacity to neutralize primary isolates of HIV-1 group M and SIVcpz viruses [19,45]. Antibodies induced by VI686 consistently lacked neutralizing activity to HIV-1 group M and SIVcpz primary isolates. However, some HIV-1 group M sera neutralized both group M and group O primary isolates, suggesting conserved neutralizing epitopes [19]. Multivariate analysis of inter- and intraclade neutralization data revealed that the neutralization spectra of the HIV-1 group O isolates form a separate cluster [46].

The principal neutralizing domains or V3 loops of CA9 and VI686 are 37 and 36 amino acids long, respectively. Comparing the C2V3 sequences of CA9 and VI686 with previously documented C2V3 regions reveals that the octamers at the tip of the loop RIGPLAWY (CA9) and KIGPMAWY (VI686) were previously documented for isolates 341Ha [34] and BCF07 [10]. HIV-1 CA9 and VI686 were of the NSI biological phenotype, even though the viruses were obtained at late-stage disease. It was previously observed for HIV-1 group M [47,48] and group O [38] that determinants of the SI phenotype are located within the V3 loop. For group O strains, a high net positive charge of the V3 loop and a positively charged amino acid at position 11 or 25 is indicative of SI/MT-2-tropic isolates, whereas a low net positive charge and a single positively charged amino acid at position 37 indicates NSI/MT-2-tropic isolates. However, the results presented in this study did not reveal an association between the presence of positively charged amino acids in the V3 loop and replication in the case of VI686 and syncytium formation in MT-2 cells.

In conclusion, we have characterized CA9 and VI686, two group O viruses from Cameroon and Gabon, respectively. Phylogenetic analysis of the *env* gene (gp160) of the nine HIV-1 group O strains documented allowed us to define a distinct subtype in HIV-1 group O, tentatively named the HIV-1 group O *env* ANT70 clade, which is currently represented by ANT70, CA9, VI686, 193Ha, 267Ha, 34lHa, and 655Ha. In addition, these seven isolates belong to the same cluster in the p24 phylogeny. VAU and MVP5180 may be representatives of other subtypes.

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