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RAPID COMMUNICATION

Various Types of HIV Mixed Infections in Cameroon

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In order to assess the incidence of HIV mixed infection as well as to clarify the molecular epidemiology of HIV in central Africa, we investigated 43 HIVs obtained from 211 Cameroonian AC, ARC, and AIDS patients in 1994 and 1995. Part of the *pol* region and part of the *env* region were phylogenetically analyzed. The genotypes observed were varied: of 43 specimens, 28 (65%) were subtype A, 1 (2%) was subtype B, 2 (5%) were subtype D, 3 (7%) were subtype F, and 2 (5%) were group O. Of the remaining 7 specimens, 3 were mixed infections with HIV-1 subtypes A and C, HIV-1 subtypes C and F, and HIV-2 subtype A and HIV-1 subtype A; 1 was a mixed infection with HIV-1 subtypes A and D and the highly divergent group O (triple infection); another 3 appeared to consist of mosaic genomes (A/G, A/E, and B/A recombinant). These data show that various types of mixed infection, such as between different subtypes of HIV-1 group M, between HIV-1 and HIV-2, and even between HIV-1 groups O and M, were confirmed at a rather high frequency (approximately 10%). The mixed infection is particularly significant where there is a greater variety of HIV-1 subtypes circulating, since it results in new genetic diversity generated by intersubtype recombination. © 1998 Academic Press

INTRODUCTION

Recently much attention has been paid to the role of mixed HIV infections in the evolution of HIV-1 and HIV-2, because their resultant intersubtype recombination may contribute substantially to the rapid emergence of HIV variants. However, the evidence for co-infection or superinfection has been rather rarely reported despite the presence of a large population of individuals with repeated exposures via sexual contacts or the sharing of contaminated needles. Several reports have so far documented mixed infection in an individual, including the existence of HIV-1/HIV-2 dual infections (10), double infection with two different subtypes of HIV-1 (2, 14), and mixed infection of multiple strains of HIV-1 subtype B (29, 3). These cases were from rather limited areas, and so, clearly, the study of mixed infection needs to be carried out on a worldwide basis.

To date, all the putative intersubtype recombinants appeared to have originated from areas where multiple subtypes are cocirculating, such as central Africa, South America, and Southeast Asia (19). In Cameroon, independent studies have reported the presence of various HIV-1 subtypes of group M (major) (20) as well as highly divergent strains (MVP5180 and ANT70) of HIV-1 group O (outlier) (11, 26, 12, 17). However, very little is known about the HIV mixed infection in Cameroon. We thought that this central African country would be suitable for assessing its incidence. Here we report our findings that mixed infection with various HIV-1 strains is not as rare as previously thought.

RESULTS

Subjects and HIV serology

Of 211 patients that took part in our study, 68 (32%) were HIV seropositive. Of these 68 patients, 60 (89%) were HIV-1 positive, 3 (4%) were dual HIV-1/2 positive, and 5 (7%) were indeterminate. None were positive for only HIV-2. DNAs from 43 samples have remained available and were sequenced for molecular characterization (Table 1). Epidemiological and clinical data relevant to these samples are summarized in Table 2. (In this study, CMRn refers to a HIV strain, where n is a number and cmrn refers to the patient from which the strain was obtained.)

The nucleotide sequence data reported in this paper were submitted to GenBank nucleotide sequence database and the following accession numbers have been assigned: the *pol* sequences (288 bp), U58157, U58158, U69207 to U69253, AF023085; the *env* sequences (approximately 390 bp), U58148, U58149, U58152, U58153, U69991 to U70014, and AF023064 to AF023084.

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Phylogenetic analysis of the HIV-1 *env* region

To obtain a molecular epidemiological overview of HIV in Cameroon, the central portion of gp120 including the V3 region was sequenced, and a phylogenetic tree was constructed (Fig. 1). The majority of HIV-1s in this country belonged to subtype A (29 of 43 specimens, 67%), and the rest belonged to various subtypes: 1 (2%) (CMR277) belonged to subtype B, 2 (5%) (CMR68 and CMR370) belonged to subtype D, 1 (2%) (CMR10) belonged to subtype E, 3 (7%) (CMR158, CMR271, and CMR304) belonged to subtype F, and 1 (2%) (CMR276) belonged to subtype G (Table 2 and Fig. 1). In addition, 2 strains (5%) (CMR275-C and CMR281-C) belonged to subtype C, whereas other strains from the same patients belonged to different subtypes (F and A) (see the "*Cases of mixed infection*..." described below). The sequences of subtypes C, D, and G had not been previously reported in Cameroon. There is no doubt that a large variety of HIV-1 subtypes, A through H (except I and J) plus group O, were found in this country. (Subtype H was previously reported in Cameroon (13).)

Figure 2 shows the deduced amino acid sequences from all the analyzed nucleotide sequences in the *env* region. For convenience of comparison, the results are compiled according to each subtype. The V3 loop (cysteine to cysteine) of all the Cameroonian strains of subtype A were composed of 35 amino acid residues. This observation is generally consistent with what has been seen in the V3 loop sequences of all the other subtypes. But CMR61-D and CMR68 (subtype D) and CMR304 (subtype F) had 34 amino acid residues, and CMR 10 (subtype E) had only 32 residues. On the other hand, CMR61-O (group O) had two types of sequences: one (CMR61-O clone 1) had 33 residues but the other (CMR61-O clone 10) had 40 residues. We also found unique tetrameric sequences at the tip of the loop. In subtype A of the African strains, GPGQ was previously shown to be a common tetrameric sequence (19). However, the sequence of CMR309 (subtype A) was GPGI which has not been reported previously, even in other subtypes. The sequences of CMR61-D and CMR370 (subtype D) were GPRQ and APGE, respectively, neither of which had been reported even in other subtypes. In addition, CMR10 (subtype E) also showed a unique tetrameric sequence, GLGH. Thus, remarkable heterogeneity was observed in the tetrameric sequences, but the GPG (Gly-Pro-Gly) motif in the V3 loop appears to be particularly dominant in Cameroonian HIV-1 group M sequences.

Phylogenetic analysis of the *pol* region

Figure 3A shows a phylogenetic tree constructed from the *pol* sequences. The 288-bp-long sequence amplified with the integrase primers is considered sufficiently representative of the whole *pol* gene (18). Unlike the *env* analysis, by which at least 10 major HIV-1 subtypes (A through J) have been described (19, 16, 1), the present *pol* analysis revealed that HIV-1 fell into five major and clearly defined subtypes: A, B, C, D, and F. The HIV-1 subtypes are genetically quite distinct, with nucleotide distances of up to 18% in the *pol* (and 30% in the *env*) in this study. In this *pol* tree, subtypes E and G were included in subtype A. Neither subtype has been previously reported as far as the *pol* sequences are concerned, and it is unknown whether they are really present. Instead, we found a new cluster (subtype F) of *pol* sequences that have not been described before. Their clustering is well supported by the high value of the bootstrap estimation. We consider that these *pol* sequences can be defined as subtype F, because both the *pol* and corresponding *env* sequences were derived from the same genome amplified by extra long (XL) PCR (data not shown). Additionally, it is noted that CMR106 is the first HIV-2 subtype A (HIV-2a) from a central African country to be identified by sequencing (Fig. 3B). It is likely that HIV-2 infection was introduced into this country in recent years (28).

Cases of mixed infection and suspected recombination

To enhance the sensitivity of detection, we used a different set of primers in addition to the standard primers (Table 1). Multiple sequences in the same genomic region were found in four individuals: HIV-1 subtypes C and F in cmr275, HIV-1 subtypes A and C in cmr281, HIV-1 group O and subtypes A and D in cmr61, and HIV-1 subtypes A and HIV-2 subtype A in cmr106 (Table 2, Figs. 1, 3A, and 3B). The coexistence of multiple sequences was observed in both the *pol* and *env* regions in cmr275, cmr281, and cmr61. (Confirmation of the existence of an HIV-2 sequence in the *env* region was not done, since two divergent sequences, HIV-1 and HIV-2, were confirmed in the *pol* analysis.) The presence of two or three different sequences in each individual clearly demonstrated that these are cases of dual infection or triple infection with HIV strains of different origins. In addition to the four above-mentioned clear cases of mixed infection, we also found two more highly probable cases of double infection with two strains belonging to the same subtype. One is cmr323, who had two types of subtype A sequences (CMR323 clone 1 and CMR323 clone 4: 12% divergence), with CMR105 (subtype A) located between these two sequences on the *env* tree. The other is cmr68, who had two types of subtype D sequences (CMR68 clone 3 (or clone 4) and CMR68 clone 11: 16% divergence) (Figs. 1 and 2).

A comparison of the *pol* and the *env* sequences revealed that most of the Cameroonian HIV-1s belonged to the same subtypes, but some of them were different (Figs. 1 and 3A). In this study, three HIV-1s (CMR276,

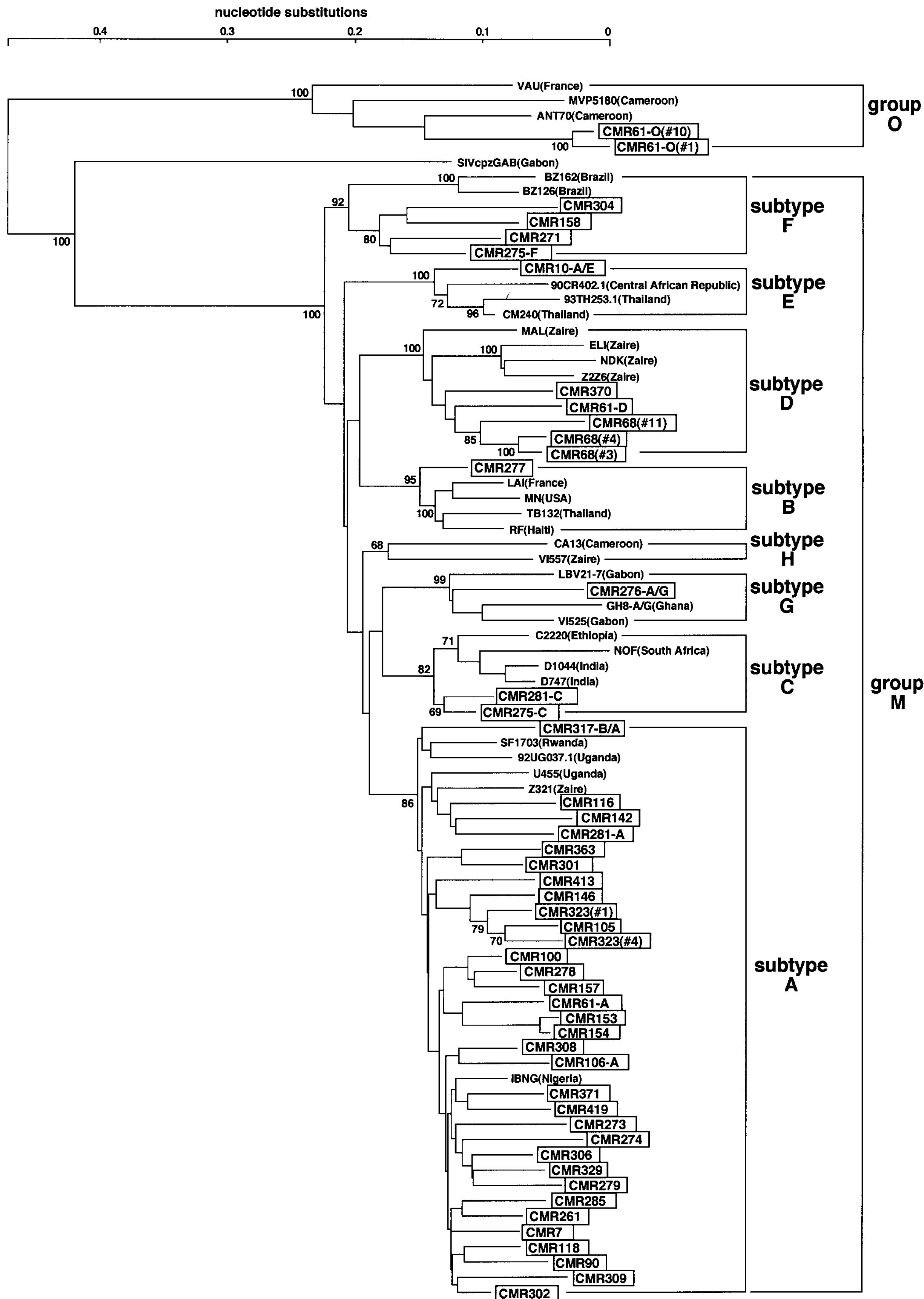

FIG. 1. A phylogenetic tree of the HIV-1 *env* sequences. The tree was constructed from part of the *env* sequences including those in the V3 region (approximately 390 bp). A few isolates belonging to group O and SIVcpzGAB (Gabon) were used as outgroups to root the tree. Subtypes are indicated by brackets, and the Cameroonian specimens are boxed.

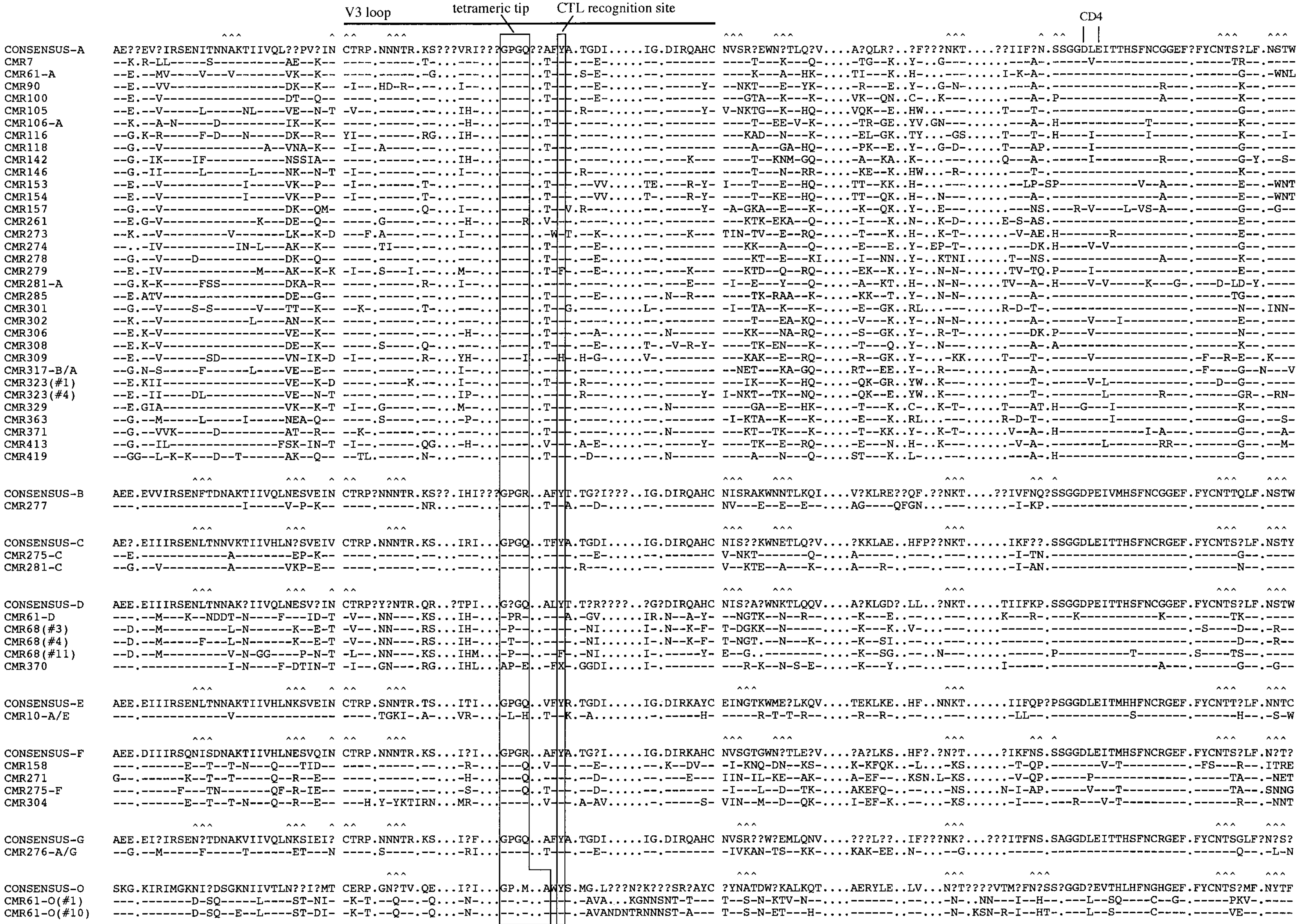

FIG. 2. Alignment of deduced amino acid sequences of the V3 region of the Cameroonian specimens. The compiled consensus amino acid sequences of subtypes A–G and group O based on the Los Alamos database are given on the top line using the single-letter codes. Cameroonian specimens are denoted by the letters CMR. Dashes (-) indicate identities with the consensus sequence. Dots (.) indicate gaps. Carets (^) show positions of potential N-linked glycosylation sites in the consensus sequence. The V3 loop is indicated with a bar.

A

nucleotide substitutions

0.2 0.1 0

100
CMR61-O
MVP5180(Cameroon)
CMR421
CMR376
ANT70(Cameroon)
group O
SIVcpzGAB(Gabon)
100
D1044(India)
86
CMR275-C
CMR281-C
C2220(Ethiopia)
D747(India)
subtype C
100
CMR304
CMR271
CMR158
CMR275-F
subtype F
ELI(Zaire)
Z2Z6(Zaire)
CMR68(#5)
CMR68(#1)
97
CMR61-D
CMR370
78
CMR317-B/A
RF(Haiti)
LAI(France)
90
CMR277
MN(USA)
subtype B
subtype D
100
GH3(Ghana)
NDK(Zaire)
CMR413
CMR323
CMR105
CMR308
GH8-A/G(Ghana)
env V3C3 subtype G
CMR116
CMR142
U455(Uganda)
90CR402.1(Central African Republic)
CMR10-A/E
CM240(Thailand)
95
93TH253.1Thailand)
NH1(Japan)
env V3C3 subtype E
84
92UG037.1(Uganda)
MAL(Zaire)
CMR329
CMR61-A
CMR106-A
CMR146
CMR90
IBNG(Nigeria)
CMR154
CMR100
CMR153
CMR278
CMR157
CMR274
CMR261
CMR273
CMR276-A/G
env V3C3 subtype G
CMR281-A
CMR371
CMR363
CMR279
CMR7
CMR118
CMR302
CMR419
CMR301
CMR309
CMR306
GH9(Ghana)
CMR285
subtype A
group M

FIG. 3. Phylogenetic trees of HIV-1 (A) and HIV-2 (B) based on part of the *pol* sequences (288 bp). The Cameroonian specimens are boxed. The phylogenetic tree (A) included two Indian isolates of subtype C (D747 and D1044), kindly provided by Dr. H. Rübsamen-Waigmann, Georg-Speyer-Haus, Frankfurt am Main, Germany, and one Japanese isolate of subtype E (NH1), kindly provided by Dr. Y. Takebe, National Institute of Infectious Diseases, Tokyo, Japan. The *pol* sequences of these isolates were determined in this study. In the HIV-2 lineages (B), subtypes C through F were not included in this tree, because their reported sequences did not overlap with ours.

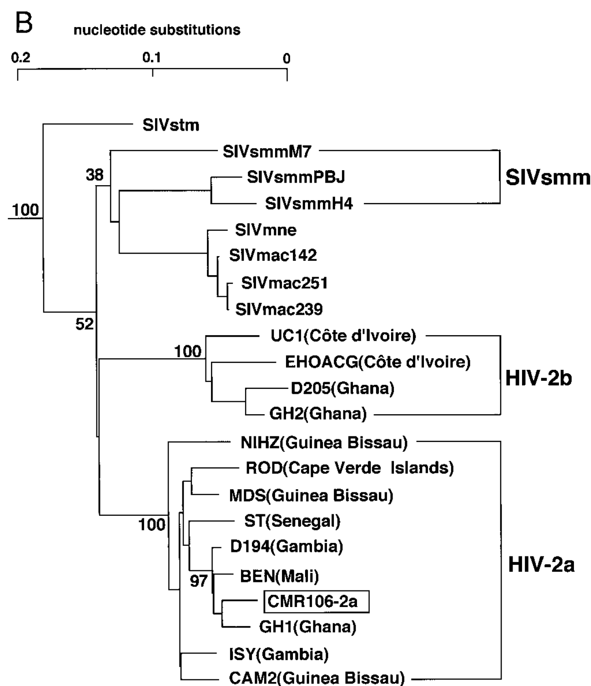

FIG. 3—*Continued*

CMR10, and CMR317) occupied different positions in two phylogenetic trees. CMR276 belonged to subtype G by its *env* sequence, whereas it was grouped into subtype A by its *pol* sequence. CMR10 exhibited a subtype A *pol* sequence in spite of its having a subtype E *env* sequence. CMR317 had an *env* sequence belonging to subtype A and a *pol* sequence belonging to subtype B. These three strains were suspected to be examples of recombination (discussed below).

DISCUSSION

To investigate the incidence of mixed infection, it is important to distinguish between mixed infection of a patient with two or more genetically distinct HIV strains and infection of a patient with an intersubtype recombinant HIV strain. Mixed infection is indicated by the coexistence of multiple sequences in a certain part of the viral genome within one patient (29). Based on this definition, it is clear that the four individuals who possessed two or more different sequences in the *pol* analysis (cmr275, cmr281, cmr61, and cmr106) were cases of mixed infection. In addition, we also found two strains of subtype A (cmr323) or subtype D (cmr68) in an individual. These two cases may be due to double infection with two different viruses, because the two sequences found in each individual showed more than 10% divergence without any strains occupying intermediate positions between the two distinct sequences. If we take into account these two more highly probable cases, the total number of mixed infections would increase to six.

On the other hand, recombination is detected when different genes, or different regions, within the same genome are placed by phylogenetic analysis into different subtypes or as distant members of established subtypes (21, 22, 6). By this definition, three subjects (CMR276, CMR10, and CMR317) were suspected to be cases of recombination. The result of CMR276 (subtype A in the *pol* and subtype G in the *env*) can be explained by an infection with an A/G recombinant. This was later confirmed through the characterization of a long sequence from the *pol* region to *env* region using XL PCR (manuscript in preparation). Likewise, the result of CMR10 (subtype A in the *pol* and subtype E in the *env*) can be interpreted as an infection with an A/E recombinant. In fact, A/E recombinants from Central African Republic and Thailand were found to possess the same pattern of mosaic structures by bootscanning (4, 8). It is likely that CMR10 is also an A/E recombinant, because we also obtained a XL PCR product (unpublished data). The result of CMR317 (subtype B in the *pol* and subtype A in the *env*) needed more careful consideration since it might be the first case of a B/A recombinant. To exclude the possibility that we missed detecting other sequences in both regions, we used a different set of primers (Table 1) that are sensitive enough for detecting each subtype in both regions. These primers were successfully used to confirm four cases of mixed infection, as mentioned above. However, only the sequence detected in the *env* region was that of subtype A. In a similar manner, only the sequence detected in the *pol* region was that of subtype B. Based on these results, we consider that cmr317 is not a case of mixed infection but a highly probable case of a B/A recombinant.

The results of the present study help to answer the following two important questions concerning mixed infections: (i) what types of mixed infection can occur *in vivo?* and (ii) what is the frequency of mixed infection? Actually, we have found various types of mixed infection: between different subtypes of HIV-1 group M, between HIV-1 and HIV-2, and even between HIV-1 groups O and M in four individuals. A case of triple infection (cmr61) with HIV-1 group O and subtypes A and D (25) was noteworthy. Our results also showed that the types of mixed infection appeared to depend on the prevalence rates of respective HIV-1 subtypes circulating in a certain population, because three of four cases consisted of combinations with HIV-1 subtype A, the most epidemic subtype in Africa.

Regarding the frequency of mixed infections, it was believed that the host's immune response played a role in protecting against superinfecting viruses, and consequently mixed infection might be rare (6). However, the present study clearly indicated that various types of mixed infection occurred at approximately 10% (4 to 6 of 43 cases), which is a rather high frequency. Indeed, a significant proportion of individ-

TABLE 1

Primers Used for Nested PCR

Primer	Sequence (5′–3′)	Location	Usage
pol region[a]			
unipol5	TGGGTACCAGCACACAAAGGAATAGGAGGAAA	3434–3765 of HIV-1LAI	Outer pair
unipol6	CCACAGCTGATCTCTGCCTTCTCTGTAATAGACC	4483–4516 of HIV-1LAI	for HIV-1/2
HIV-1pol3	TAAAAGGAGAAGCCATGCATGGACAAGTAGA	3945–3975 of HIV-1LAI	Outer pair
HIV-1pol4	TCACCTTTCCAGAGGAGCTTTGCTGGTCCTTTCC	4515–4548 of HIV-1LAI	for HIV-1
HIV-2pol3	AAATGCAGAACTAGGCACTTGGCAAATGG	4218–4245 of HIV-2GH1	Outer pair
unipol6	CCACAGCTGATCTCTGCCTTCTCTGTAATAGACC	4727–4760 of HIV-2GH1	for HIV-2
unipol1	AGTGGATTCATAGAAGCAGAAGT	4052–4074 of HIV-1LAI	Inner pair
unipol2	CCCCTATTCCTTCCCCTTCTTTTAAAA	4363–4388 of HIV-1LAI	for HIV-1/2
env region[b]			
M5	CCAATTCCCATACATTATTGTGCCCCAGCTGG	6451–6482 of HIV-1LAI	Outer pair
M10	CCAATTGTCCCTCATATCTCCTCCTCCAGG	7225–7254 of HIV-1LAI	for group M
M7	GTCCAAAGGTATCCTTTGAGCCAATTCCCATAC	6431–6463 of HIV-1LAI	Outer pair
M12	GGTGGGTGCTACTCCTAGTGGTTCAATTT	7286–7314 of HIV-1LAI	for group M
O7	GAGCCCATTCCCATACACTATTGTGCTCC	6889–6917 of HIV-1MVP5180	Outer pair
O8	TTCTCCATATATCTTTCATGTCTCCCCCT	7692–7720 of HIV-1MVP5180	for group O
M3	GTCAGCACAGTACAATGIACACATGG	6541–6566 of HIV-1LAI	Inner pair
M8	TCCTTGGATGGGAGGGGCATACATTGC	7114–7140 of HIV-1LAI	for group M
O3	CACATGGCATCAAGCCAACAGTAAGTAC	7001–7028 in HIV-1MVP5180	Inner pair
O4	TAGGTTTCCTGGGATGGGAGGTGCATA	7582–7608 in HIV-1MVP5180	for group O

[a] Universal HIV-1/2 primers (unipol5 and unipol6) were used in the standard first round. In addition, we used HIV-1-specific primers (HIV-1pol3 and HIV-1pol4) and HIV-2-specific primers (HIV-2pol3 and unipol6) in the first round.
[b] HIV-1 group M-specific primers (M5 and M10) were used in the standard first round. In cases of mixed infection or recombination, we also used HIV-1 group M-specific primers (M7 and M12) in the first round.

uals may be co-infected (or superinfected) with various HIV strains, where there is a greater variety of HIV-1 subtypes circulating.

The high frequency of mixed infection appears to correlate with the high incidence of recombinant viruses. Recently, it was noted that 5 to 10% of HIV-1 strains that have been at least partially sequenced consisted of mosaic genomes (22, 6). In this study, three HIV-1s (CMR10, CMR276, and CMR317) were suggested to possess intersubtype mosaic genomes. This incidence (3 of 43, 7%) is consistent with that in the database and may be a consequence of the high frequency of mixed infection. Our findings that mixed infection with divergent HIV-1 strains is not as rare as previously thought are important, since such information provides a basis for developing AIDS vaccines as well as for predicting the global evolution of HIV in the future.

MATERIALS AND METHODS

Subjects

Study populations were recruited in selected Cameroonian hospitals (Bertoua, Batouri, Yaoundé, Douala, Akonolinga, Awaé, and Sangmélima) in 1994 and 1995. The subjects enrolled in this study were selected from 211 patients who were suspected of having sexually transmitted disease (STD), tuberculosis (TB), and AIDS. Patients were classified as being an asymptomatic carrier (AC), having AIDS-related complex (ARC), or having full-blown AIDS. Basic sociodemographic information as well as an assessment of risk factors for acquiring HIV infection were collected for each patient. After obtaining verbal informed consent, blood was collected in a citrate-containing bag (ACD anticoagulant solution). A syphilis serology was also performed and free treatment was provided when necessary. Basic drugs (antifungal, antibiotics etc.) were given when needed.

Serological tests

Sera were screened by a particle agglutination (PA) test (Serodia-HIV, Fujirebio, Tokyo, Japan). All reactive specimens were confirmed by a Western blotting (WB) assay (New Lavblot HIV-1 and HIV-2; Sanofi Diagnostic Pasteur, Marnes-la-Coquette, France). Dually reactive sera were differentiated using a peptide-based assay (Peptilav HIV-1 and HIV-2; Sanofi Diagnostic Pasteur) and judged as HIV-1 or HIV-2 positive.

TABLE 2

Epidemiological and Clinical Information for Study Participants

Subject	Age (years)	Sex[a]	Virus isolation	Disease status[b]	Blood transfusion	IDU[c]	Year	Serology	Genotype	
									pol	*env*
cmr7	55	F	Isolate	ARC	—[d]	—	1994	HIV-1	A	A
cmr90	34	M		AC	—	—	1994	ID[e]	A	A
cmr100	16	F		AC	—	—	1994	HIV-1	A	A
cmr105	21	F		AC	—	—	1994	HIV-1	A	A
cmr116	28	F		AC	—	—	1994	HIV-1	A	A
cmr118	34	M	Isolate	AIDS	—	—	1994	HIV-1	A	A
cmr142	24	M		AC	—	—	1994	HIV-1	A	A
cmr146	32	M		AC	—	—	1994	HIV-1	A	A
cmr153	35	M		AIDS	—	—	1994	HIV-1	A	A
cmr154	40	F		AC	—	—	1994	HIV-1	A	A
cmr157	49	M		AC	—	—	1994	HIV-1	A	A
cmr261	38	M	Isolate	AIDS	—	—	1994	HIV-1	A	A
cmr273	NA[f]	NA		NA	NA	NA	1994	HIV-1	A	A
cmr274	NA	NA		NA	NA	NA	1994	HIV-1	A	A
cmr278	NA	NA		NA	NA	NA	1994	HIV-1	A	A
cmr279	NA	NA		NA	NA	NA	1994	HIV-1	A	A
cmr285	NA	NA		NA	NA	NA	1994	HIV-1	A	A
cmr301	33	M	Isolate	ARC	—	—	1994	HIV-1	A	A
cmr302	27	M	Isolate	ARC	—	—	1994	HIV-1	A	A
cmr306	NA	F	Isolate	AC	—	—	1994	HIV-1	A	A
cmr308	NA	M	Isolate	AIDS	—	—	1994	HIV-1	A	A
cmr309	NA	M	Isolate	ARC	—	—	1994	HIV-1	A	A
cmr323	NA	NA		NA	NA	NA	1994	HIV-1	A	A
cmr329	NA	NA		NA	NA	NA	1994	HIV-1	A	A
cmr363	49	M		ARC	—	—	1995	HIV-1	A	A
cmr371	50	M		AIDS	—	—	1995	HIV-1	A	A
cmr413	NA	F		AC	—	—	1995	HIV-1	A	A
cmr419	44	F		ARC	—	—	1995	HIV-1	A	A
cmr277	NA	NA		NA	NA	NA	1994	HIV-1	B	B
cmr68	45	F		AC	—	—	1994	HIV-1	D	D
cmr370	NA	NA		AC	—	—	1995	HIV-1	D	D
cmr158	34	M	Isolate	AIDS	—	—	1994	HIV-1	F	F
cmr271	NA	NA		NA	NA	NA	1994	HIV-1	F	F
cmr304	NA	M	Isolate	ARC	—	—	1994	HIV-1	F	F
cmr376	NA	M		ARC	—	—	1995	dual HIV	O	NA
cmr421	19	M		AC	—	—	1995	HIV-1	O	NA
cmr10	34	F		AIDS	—	—	1994	HIV-1	A	E
cmr276	NA	NA		NA	NA	NA	1994	HIV-1	A	G
cmr317	NA	NA		NA	NA	NA	1994	HIV-1	B	A
cmr281	NA	NA		NA	NA	NA	1994	HIV-1	"A, C"[g]	"A, C"
cmr275	NA	NA		NA	NA	NA	1994	HIV-1	"C, F"	"C, F"
cmr106	25	F		AC	—	—	1994	dual HIV	"A, 2a"[h]	"A, ND"[i]
cmr61	22	F		ARC	—	—	1994	HIV-1	"O, A, D"	"O, A, D"

[a] M, male; F, female.
[b] AC, asymptomatic carrier; ARC, AIDS-related complex.
[c] IDU, injection drug user.
[d] —No history.
[e] ID, indeterminate.
[f] NA, information not available.
[g] "A, C", mixed-infection with HIV-1 subtypes A and C; the contents of mixed-infection are expressed likewise in the following specimens.
[h] 2a, HIV-2 subtype A.
[i] ND, not done.

Virus isolation

Peripheral blood mononuclear cells (PBMCs) from confirmed (or indeterminate) HIV-positive patients were separated by Ficoll gradient sedimentation and cocultivated with virus-free human PBMCs or human T-lymphoid cell lines (Molt-4 clone 8 (15) or M8166 (5)). Cultures were periodically checked by immunofluorescence assay (IFA) for expression of viral antigens using autologous and reference anti-HIV-1 and/or -2 sera. Antigen-positive cultures were subsequently examined for virus production by measuring reverse transcriptase (RT) activity in the supernatants as described (27). Regardless of the results of virus isolation, primary uncultured PBMCs were analyzed for HIV by PCR.

PCR and DNA sequencing

Chromosomal DNA was extracted from PBMCs using glass milk powder (Prep-A-Gene DNA purification kit; Bio-Rad, Hercules, CA), and the following two regions of proviral DNA were amplified by nested PCR. One region (corresponding to 4075–4362 nt in HIV-1LAI) was part of the *pol* open reading frame that encodes integrase. The other region (corresponding to 6613–6999 nt in HIV-1LAI) was part of the gp 120 gene of covering one-fourth of the C2 region, the V3 domain, and the whole length of the C3 region. The primers used are listed in Table 1. PCR was performed as previously described (23, 24). Purified PCR products (339 bp long in the *pol* region and approximately 600 bp long in the *env* region) were subcloned into the *Sma*I site of pUC119. DNA sequencing was carried out by the dideoxy chain-termination method using an automated DNA sequencer (373A; Applied Biosystems, Foster City, CA). At least seven plasmid clones were sequenced to obtain the consensus sequence.

Phylogenetic analysis

DNA sequences were aligned by the ODEN program package at the National Institute of Genetics, Mishima, Japan. Nucleotide substitutions were estimated by the six-parameter method (9), and then the phylogenetic tree was constructed by the neighbor-joining method, and its reliability was estimated by 100 bootstrap replications (7).

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