JOURNAL OF VIROLOGY, Oct. 1995, p. 6122–6130 Vol. 69, No. 10
0022-538X/95/$04.00+0

Genetic Analysis of Human Immunodeficiency Virus Type 1 Strains from Patients in Cyprus: Identification of a New Subtype Designated Subtype I

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Received 6 March 1995/Accepted 22 June 1995

DNA sequences encoding the C2 to V3 region of envelope glycoprotein gp120 of human immunodeficiency virus type 1 (HIV-1) were amplified by PCR from uncultured peripheral blood mononuclear cells obtained from 24 of 25 HIV-1-seropositive patients from Cyprus. By using a heteroduplex mobility assay (HMA), all amplified products were studied genetically and compared with 16 previously characterized HIV-1 strains belonging to subtypes A through F. HMA results revealed that HIV-1 gp120 sequences from 15 of our patients were of subtype B of HIV-1, whereas one isolate was of subtype C. However, gp120 sequences from eight patients had no obvious similarities to the known subtypes as defined by HMA. DNA sequencing and phylogenetic analyses of molecular clones confirmed the HMA results and placed the eight undefined HIV-1 isolates into three distinct genetic clusters. On the basis of branch topology and lengths of the phylogenetic tree, we conclude that one group consisting of three clones from two patients represents a new HIV-1 *env* subtype, which we have termed subtype I. The remaining two sequence clusters, consisting of five sequences from four patients and two sequences from two other patients, are distally related to subtypes A and F. These data demonstrate the extensive heterogeneity of HIV-1 in Cyprus, including the presence of a new subtype.

The global molecular epidemiology of human immunodeficiency virus type 1 (HIV-1) is being studied through the combined efforts of the World Health Organization (WHO) and many other groups worldwide. Phylogenetic analysis of HIV-1 sequences obtained from many countries has revealed eight distinctive phylogenetic subtypes among the isolates within the major group, M (1–5, 12, 14, 17, 18, 21–24, 26, 30–32, 34, 35, 36, 37, 39, 42–44, 47, 55, 56). The existence of these eight subtypes, named A through H, has many important implications for the global evolution of HIV-1 and future vaccine development.

HIV-1 was initially detected in Cyprus in the mid-1980s, at about the time of its first appearance in Asia (55) and countries of the eastern Mediterranean region (51). The first patient with AIDS in Cyprus, a young woman who lived in the United States, was diagnosed in 1986 and died in 1987 (13). The reported prevalence of HIV-1 infection in Cyprus is lower (about 1 per 5,000) than in European countries and United States and is similar to the prevalence in other countries in the eastern Mediterranean region (33, 51). The central geographic location of Cyprus (located between Europe, Africa, and the Middle East), the high frequency of tourist visits from different continents, and the large number of Cypriots living abroad are factors that led us to suspect that HIV-1 isolates from Cyprus might be unusually heterogeneous and may include members of some as yet undiscovered subtype(s). We have therefore determined the extent of genetic diversity among HIV-1 sequences from 25 Cypriots infected with HIV-1 and compared these sequences with HIV-1 sequences previously obtained from diverse geographic locations.

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MATERIALS AND METHODS

Patients. Blood samples were obtained from 25 HIV-1-seropositive patients from the AIDS Clinic, Nicosia General Hospital, Nicosia, Cyprus, in February 1994 with the informed consent of all participants and the approval of the Ministry of Health. All subjects were Greek-Cypriot citizens living permanently in Cyprus at the time of study, although a number of them reported traveling or living abroad in the past. The HIV-1 serostatus of each study subject was previously established by commercial immunoassay and confirmed by Western blotting (immunoblotting). The study subjects comprised of 6 patients with AIDS or AIDS-related complex and 19 with asymptomatic infection. Two of the patients with AIDS died during the course of the study. All symptomatic patients were receiving zidovudine when blood was drawn. A description of the clinical profile of each subject is presented in Table 1. All blood samples were processed at the Aaron Diamond AIDS Research Center after a 1-day delay in transport.

HIV-1 isolation. Patients' peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque gradient sedimentation. Plasma samples were obtained from heparinized blood after sedimentation at 3,000 × *g* for 15 min at room temperature (20). HIV-1 RNA levels in plasma were determined by the branched DNA signal amplification method (Quantiplex HIV-RNA assay; Chiron Corporation, Emeryville, Calif.) as previously described (7). Both plasma and PBMC were used for HIV-1 isolation as previously described (20). The remaining PBMC and plasma samples were cryopreserved at −80°C until further use.

PCR. DNA was isolated by guanidinium thiocyanate extraction from 3×10^6 uncultured PBMC (DNA/RNA isolation kit; United States Biochemical Corp., Cleveland, Ohio). HIV-1 DNA sequences encoding approximately 530 bp of the gp120 C2 to V3 region were amplified from each sample by nested PCR. Primers used in the first round of PCR were ED5 (5′ ATGGGATCAAAGCCTAAAGC CATGTG at positions 6553 to 6578 of the pNL4-3 sequence) (2) and ED12 (5′ AGTGCTTCCTGCTGCTCCCAAGAACCCAAG at positions 7772 to 7801) (11); inner primers were LK1 (5′-CCAATTCCCATACATTATTGTGCCCCG GCTGG at positions 6850 to 6879) and LK2 (5′-TTACAGTAGAAAAATTC CCCTCCACAATTAAA at positions 7330 to 7361). The rationale for designing a new set of second-round PCR primers in this study was to minimize the length variation in the target gp120 sequence that could complicate the genetic analysis. Negative PCR controls included PBMC DNA from healthy HIV-1-seronegative individuals, isolated and amplified under identical experimental conditions. Reference plasmids containing HIV-1 gp120 clones of subtypes A (RW20, IC144, and SF170), B (BR20, TH14, and SF162), C (CMA959, ZM18, and IN868), D (UG21, UG38, and UG46), E (TH22 and TH06), and F (BZ162 and BZ163) were provided by E. Delwart, C. Cheng-Mayer, and the WHO Network for HIV-1 Isolation and Characterisation.

In the first round of PCR, approximately 1 μg of patient PBMC DNA or

TABLE 1. Clinical and epidemiological information for study patients[a]

Patient	Sex[b]	Age (yr)	Yrs of infection[c]	Clinical status[d]	CD4 (cells/mm^3)	HIV-1 isolation[e]		Plasma HIV-1 RNA[f] (copies/ml)	Epidemiological information[g]
						Plasma	PBMC		
HO02	F	51	6	ASM	200	−*	−*	−	Born and lived in Zambia; husband died of AIDS
HO04	M	24	4	ASM	516	−*	+	−	Lived abroad
HO10	M	38	10	ASM	NA	−*	−*	−	Heart operation and blood transfusion in U.K. in 1984
HO11	M	29	7	SM	277	−*	+	−	Lived in Greece; homosexual
HO12	M	26	3	ASM	NA[h]	−*	+	−	Infected in Cyprus; homosexual
HO16	M	29	6	SM	60	−*	+	3.16	Born and lived in Zaire; bisexual
HO17	F	35	2	SM	2	−*	+	−	Lived in U.K.; HIV-1-infected husband (HO34); died in May 1994
HO21	M	38	4	ASM	743	+*	+	2.29	Infected in Greece; bisexual
HO25	M	34	6	ASM	650	−*	+	4.65	Lived in U.S.; heterosexual
HO27	M	20	2	ASM	709	−*	+	−	Infected in Cyprus; homosexual
HO28	F	39	1	ASM	430	−*	−*	−	Infected in Cyprus; homosexual
HO29	M	49	2	ASM	420	−*	−*	−	Infected in Cyprus; heterosexual
HO31	F	24	5	ASM	NA	−*	−*	−	Lived in Greece; IVDU; heterosexual partner of HO32
HO32	M	35	5	ASM	NA	−*	+	−	Lived in Greece; IVDU; heterosexual partner of HO31
HO34	M	36	2	ASM	410	−*	+	2.46	Lived in U.K.; wife (HO17) died of AIDS
HO39	M	37	5	ASM	470	−*	+	1.23	Lived in Greece; heterosexual
HO40	F	29	6	SM	92	−*	+	2.66	Lived in U.S.; heterosexual
HO42	F	45	4	SM	80	+*	+	9.43	Infected in Cyprus; heterosexual partner HO34; died in August 1994
HO43	M	36	6	SM	396	+	+	13.30	Lived in U.K. and Greece; homosexual
HO44	F	32	1	ASM	1,136	−*	−	−	Infected in Cyprus; heterosexual partner HO16
HO45	M	32	1	ASM	453	−*	+*	−	Infected in Cyprus; heterosexual
HO46	M	32	4	ASM	107	−*	−*	−	Lived in U.K.; heterosexual partner died of AIDS
HO48	M	22	11	ASM	276	−*	−*	−	Blood product (factor VIII) recipient in Greece
HO49	M	2	3	ASM	1,211	−*	+*	−	HIV-1 infected since birth; mother (HO17) died of AIDS
HO50	M	32	1	ASM	315	−*	+	−	Lived in Greece; homosexual partner of a person with AIDS

[a] Blood was drawn from all patients on 14 February, 1994.
[b] F, female; M, male.
[c] Indicates the first known positive HIV antibody test.
[d] ASM, asymptomatic; SM, symptomatic.
[e] Performed as described in Materials and Methods. +, positive (>200 pg of p24 antigen per ml); − negative (<5 pg of p24 antigen per ml). Asterisks indicate 3-week culture incubation period; all other cultures were incubated for 2 weeks.
[f] Negative plasma HIV-1 RNA values (−) indicate concentrations of less than 10^4 HIV-1 RNA copies per ml.
[g] Obtained from personal interviews. U.K., United Kingdom; U.S., United States; IVDU, intravenous drug user.
[h] NA, not available.

reference plasmid DNA was used, as were 20 to 40 pmol of ED5 and ED12 primers, 200 μM each dGTP, dATP, dTTP, and dCTP, 5 U of *Taq* DNA polymerase (Perkin-Elmer Cetus, Emeryville, Calif.), 2 μM $MgCl_2$, 50 mM KCl, and 10 mM Tris-Cl (pH 8.3) in a 100-μl volume. DNA amplifications were carried out in a Perkin-Elmer 9600 thermocycler. The thermocycling conditions were 1 cycle at 94°C for 2 min, 35 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1.75 min, and 1 cycle at 72°C for 7 min. In the second round of PCR, 5-μl samples of primary reaction products were used, along with 10 pmol of LK1 and LK2 primers and 10 μCi of [α-^{32}P]dATP. All other reaction components and amplification conditions were identical to those described for the first round of PCR.

HMA. PCR products from study subjects were genetically compared with 16 previously characterized HIV-1 strains by a heteroduplex mobility assay (HMA) (10, 11). DNA heteroduplexes were formed by thermal denaturation and reannealing of 0.1 μl of radiolabeled patient DNA and 10 μl (1:100 molar ratio) of unlabeled known subtype reference DNA from the second-round PCR products. Melting and annealing conditions were 94°C for 2 min and 22°C for 5 min, respectively, followed by rapid cooling on ice. The electrophoretic mobilities of all generated homoduplex and heteroduplex DNA fragments were determined in 5% (40:0.8 acrylamide/bisacrylamide), 1.5-mm-thick, neutral (88 mM Tris-borate, 2 mM EDTA [pH 8.0]) polyacrylamide gels in an SE 400 vertical electrophoresis apparatus (Hoefer Scientific) for 3 h at 250-V constant voltage. During electrophoresis, the current was 26 ± 2 mA, power was 7 ± 1 W, and the gel temperature was maintained at 34 ± 2°C.

Cloning and DNA sequence. Amplified products from the second-round PCR were cloned into the pCR II plasmid and amplified in *Escherichia coli* INVαF′ (TA Cloning kit; Invitrogen Corp., San Diego, Calif.) according to the manufacturer's specifications. Single bacterial colonies harboring the plasmid with the correct gp120 DNA insert were grown in 250-ml bacterial cultures, and the plasmid was isolated from transfected cells by alkaline lysis (49). Supercoiled plasmid was further purified by CsCl-ethidium bromide equilibrium density gradient sedimentation, dialyzed against 10 mM Tris HCl–0.1 mM EDTA (pH 8.0), and analyzed by 1% neutral (88 mM Tris-borate, 2 mM EDTA [pH 8.0]) agarose gels. The DNA sequence of each clone was determined by sequencing both complementary strands in two separate reactions, using alkaline-denatured plasmid as the template and LK1 and LK2 as primers. DNA sequence reactions were performed by the dideoxy-chain termination method, using Sequenase 2.0 polymerase (United States Biochemical). Premature chain terminations due to secondary structures of the template were minimized by maintaining the temperature at 45°C during termination reactions. Band compressions were avoided by replacing dGTP with 7-deaza-dGTP and by maintaining the gel temperature at 50 ± 2°C during electrophoresis.

Phylogenetic tree analyses. We used DNA sequence alignment, distance calculation, and phylogenetic tree construction programs from the Wisconsin Sequence Analysis Package (Genetic Computer Group, Inc.). DNA sequences were aligned by the progressive alignment method previously described (16, 19) in the PILEUP program run with default conditions. Aligned DNA sequences were edited to retain only the regions which have homologs in other aligned sequences. Pairwise distance matrices were calculated by algorithms described by Jukes and Cantor (see reference 52), Tajima and Nei (53), and Kimura (27), using the DISTANCES program, and phylogenetic trees were constructed by the GROWTREE program, which is based on the neighbor-joining method using algorithms originally described by Saitou and Nei (48). Bootstrapping analyses and final tree construction were carried out with PHYLIP (15). GenBank accession numbers for control reference sequences in phylogenetic analyses are as follows: A-SF170, M66533; A-UG37, UO9127; A-Z321, M15896; A-UG37, UO9127; B-SF162, M38428; B-SF2, KO2007; B-TH14, UO8801; C-ZM18, L22954; C-IND868, UO7103; C-BR25, UO9133; C-CMA959, UO8453; D-UG21 UO8804; D-UG46, UO8809; D-ELI, KO3454; E-CM243, LO3703; E-TH22, UO9131; F-BZ162, L22084; F-BZ163, U9665; F-R24, L19573; F-R34, L19576; G-LBV21-7, U9664; G-VI525, U9665; H-CA13, UO9667; H-VI557, UO9666; and A/C recombinant ZAM184, L22955 (46).

Nucleotide sequence accession numbers. GenBank accession numbers for the Cypriot sequences obtained in this study are as follows: HIVCYHO021, U28321; HIVCYHO024, U28661; HIVCYHO164, U28662; HIVCYHO042, U28663; HIVCYHO111, U28664; HIVCYHO173, U28665; HIVCYHO044, U28666;

HIVCYHO211, U28667; HIVCYHO251, U28668, HIVCYHO271, U28669; HIVCYHO281, U28670; HIVCYHO294, U28671; HIVCYHO311, U28672; HIVCYHO321, U28685; HIVCYHO322, U28673; HIVCYHO341, U28674; HIVCYHO342, U28719; HIVCYHO391, U28675; HIVCYHO401, U28676; HIVCYHO422, U28677; HIVCYHO433, U28678; HIVCYHO441, U28679; HIVCYHO451, U28680; HIVCYHO464, U28681; HIVCYHO481, U28682, HIVCYHO491, U28683, and HIVCYHO503, U28684.

RESULTS

Clinical and epidemiological features of study subjects. The study group consisted of 25 HIV-1-seropositive patients from the AIDS Clinic of Nicosia General Hospital (NGH) in Cyprus. Each patient is identified by a hospital registration number in sequential order of admission to the clinic. The study patients represent 50% of the total number of HIV-1-infected persons monitored in this clinic since 1987. Their general clinical features are summarized in Table 1. Their ages range from 2 to 51; 18 are males and 7 are females. Six patients have AIDS or symptomatic infection, whereas nineteen have asymptomatic infections. Their $CD4^+$ lymphocyte counts range from 2 to 1,136 cells/mm^3. Nine were infected by homosexual contact; eleven were infected by heterosexual contact; two were infected by contaminated blood or blood product; two were infected by intravenous drug use; and one child was infected by vertical transmission. The minimum duration of infection in all subjects except for HO10 was established by the date of the first positive test for antibodies against HIV-1. The duration of infection for HO10 was determined based on the year he had a heart operation and blood transfusion in the United Kingdom.

Epidemiological features among the 25 subjects were diverse. Seven subjects were infected in Cyprus: three men by homosexual contact, and two men and two women by heterosexual contact. Eight subjects were presumably infected in Greece: two by intravenous drug use; four men by homosexual contact; one man by heterosexual contact; and one man by transfusion of contaminated factor VIII. One woman was infected in Zambia, and one man was infected in Zaire. Three subjects were infected in the United States: one man and one woman heterosexually and one man homosexually. Five people were presumably infected in the United Kingdom: one man heterosexually; one man by blood transfusion; and a three-member family who reported living in the United Kingdom whose route of infection is unknown.

Virus isolation from patients' PBMC and plasma. HIV-1 isolation from the PBMC and plasma of each subject was attempted by established methods (8, 9, 20). HIV-1 was isolated from PBMC of all symptomatic subjects and 10 asymptomatic subjects and from the plasma of 3 subjects, HO43, HO42, and HO21 (Table 1). Particle-associated HIV-1 RNA was detectable by using the branched DNA assay in the plasma of eight symptomatic subjects (HO16, HO40, HO42, and HO43) and four asymptomatic subjects (HO21, HO25, HO34, and HO39), in amounts ranging from 12,300 to 130,000 copies per ml. The relatively high number of HIV-1-negative cultures may be due to the lower viability of PBMC caused by the delay in transporting the blood from Cyprus to New York.

PCR and HMA. Uncultured PBMC from all subjects were HIV-1 positive by PCR, except for subject HO10. All PCR attempts to amplify HIV-1 DNA from subject HO10, either by using a number of available PCR primers (10, 11, 57) or by extracting DNA from up to 10^8 PBMC, were negative. However, plasma from this subject was repeatedly positive for antibodies against HIV-1, indicating that he was infected. The negative PCR combined with the negative virus isolation (Table 1) may be a reflection of low viral load, as described for long-term survivors (6), or may indicate infection with a particularly diverse strain that is not amplifiable by the available PCR primers.

All HIV-1 PCR products, approximately 530 bp containing the C2 to V3 region of *env*, were genetically examined by HMA (10, 11). Radiolabeled PCR-amplified products from each subject were individually mixed with a 100-fold molar excess of corresponding PCR products from 16 previously sequenced HIV-1 isolates from subtypes A through F (41). The resultant DNA heteroduplexes were analyzed on the basis of their electrophoretic mobility in neutral polyacrylamide gels, and the genetic relationships between each viral sequence and reference HIV-1 sequences were compared. Figure 1 shows examples of HMA results from eight subjects. For subject HO02, the prominent dark bands migrating between single-stranded DNA and homoduplex positions correspond to heteroduplexes formed between HO02 viral sequences and subtype C reference sequences. This finding suggests that the genetic divergence between HO02 and subtype C sequences is less than between sequences from the other subtypes. For subjects HO04, HO11, HO12, HO21, HO25, HO27, and HO28, fast-migrating heteroduplexes with subtype B reference sequences were evident. On the basis of these findings, we concluded that the HIV-1 isolate from subject HO02 belongs to subtype C and the HIV-1 isolates from subjects HO04, HO11, HO12, HO21, HO25, HO27, and HO28 belong to subtype B. HIV-1 sequences were similarly assigned to subtype B by HMA for subjects HO29, HO39, HO40, HO43, HO45, HO46, HO48, and HO50 (data not shown).

In contrast, sequences from subjects HO17, HO31, HO32, HO34, HO42, HO44, and HO49 could not be classified into HIV-1 subtypes A to F by HMA. The HMA results from six of these patients are shown in Fig. 2. The absence of any high-mobility heteroduplexes (migrating between single-stranded DNA and homoduplex positions) indicates a high genetic divergence between HIV-1 sequences from these subjects and reference control sequences from subtypes A through F. We estimate the average genetic divergence between sequences from this cohort and reference sequences to be greater than 25 to 30%.

DNA sequencing and phylogenetic analysis. The epidemiologic relationships between all Cypriot sequences derived by HMA were reconfirmed and further analyzed by nucleotide phylogenetic analysis. HIV-1 PCR fragments from each subject were cloned into a pCR cloning plasmid, several clones from each subject were amplified, and 27 *env* inserts (~450 bp) from the C2 to V3 region were sequenced. A phylogenetic tree (Fig. 3) was constructed on the basis of 253 unambiguously aligned positions of Cypriot sequences. In addition to the Cypriot sequences, 23 previously sequenced HIV-1 isolates from diverse global locations, encompassing all eight known subtypes (A through H), were also included in the analysis. Reference sequences include three from subtype A (SF170, Rwanda; UG37, Uganda; and Z321, Zaire), three from subtype B (SF162 and SF2, United States; TH14, Thailand), four from subtype C (BR25, Brazil; CMA959, Malawi; ZM18, Zambia; and IND868, India), three from subtype D (ELI, Zaire; UG21 and UG46, Uganda), two from subtype E (CM243 and TH22, Thailand), four from subtype F (BZ162 and BZ163, Brazil; R24 and R34, Romania), two from subtype G (LBV21-7 and VI525, Gabon), and two from subtype H (CA13, Cameroon; and VI557, Gabon). Several more sequences not included in our phylogenetic tree (Fig. 3) were further used in independent phylogenetic analyses performed by G. Myers and colleagues at Los Alamos National Laboratory.

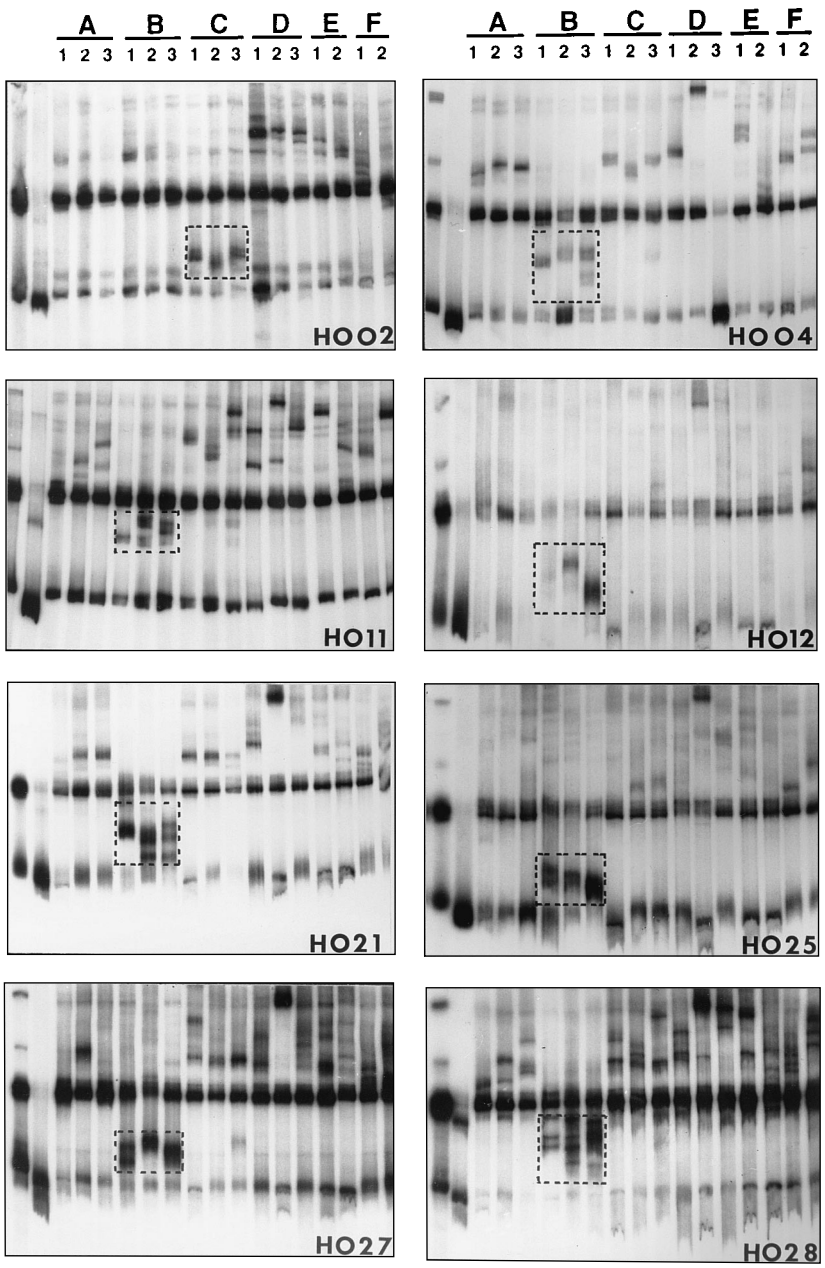

FIG. 1. Radiographs of HMA from eight representative patients infected with HIV-1 strains of known subtypes (A through F). The patient's identification number is indicated on the right bottom corner of each gel. The first and second control lanes of each gel are radiolabeled patient DNA in buffer and homoduplex double-stranded DNA formed by thermal denaturation and reannealing of radiolabeled patient DNA and a 100-fold molar excess of unlabeled patient DNA, respectively. The gel migration position of single-stranded DNA is shown in the middle of the first lane, and homoduplex double-stranded DNA is shown at the bottom of both lanes. The remaining 16 lanes of each gel are heteroduplexes formed by thermal denaturation and reannealing of radiolabeled patient DNA and about a 100-fold molar excess of unlabeled known subtype (A through F) reference DNA. The numbers under each subtype represent the reference strains given in parentheses: A (RW20, IC144, and SF170), B (BR20, TH14, and SF162), C (CMA959, ZM18, and IN868), D (UG21, UG38, and UG46), E (TH22 and TH06), and F (BZ162 and BZ163). For example, the bands migrating between the single-stranded DNA and homoduplex double-stranded DNA positions, enclosed by a box, represent the predominant fast-migrating heteroduplexes revealing the genetic similarity of patient HO02 sequence with the three reference strains in subtype C.

Nine distinct subtypes, eight previously known and a new one, were identified in the phylogenetic tree in Fig. 3. Subtype assignments were made according to established criteria based on (i) branch arrangements or topology, (ii) approximate equidistant lineages, and (iii) sequences obtained from two or more subjects (29, 40, 50). According to the phylogenetic tree in Fig. 3, Cypriot sequences fell into five subtypes: subtype A, 5 sequences; subtype B, 15 sequences; subtype C, 2 sequences; subtype F, 2 sequences; and the new subtype I, 3 sequences. The average genetic distances among Cypriot sequences within each subtype are presented in Table 2. It is notable that Cypriot sequences in subtype B (60% of subjects) have a relatively high average of intrasubtype genetic divergence of 15.6%, with a range of 0.4 to 25.6%. This finding suggests that subtype B, the predominant subtype in Europe and in the United States, was transmitted to Cyprus by multiple sources, a conclusion

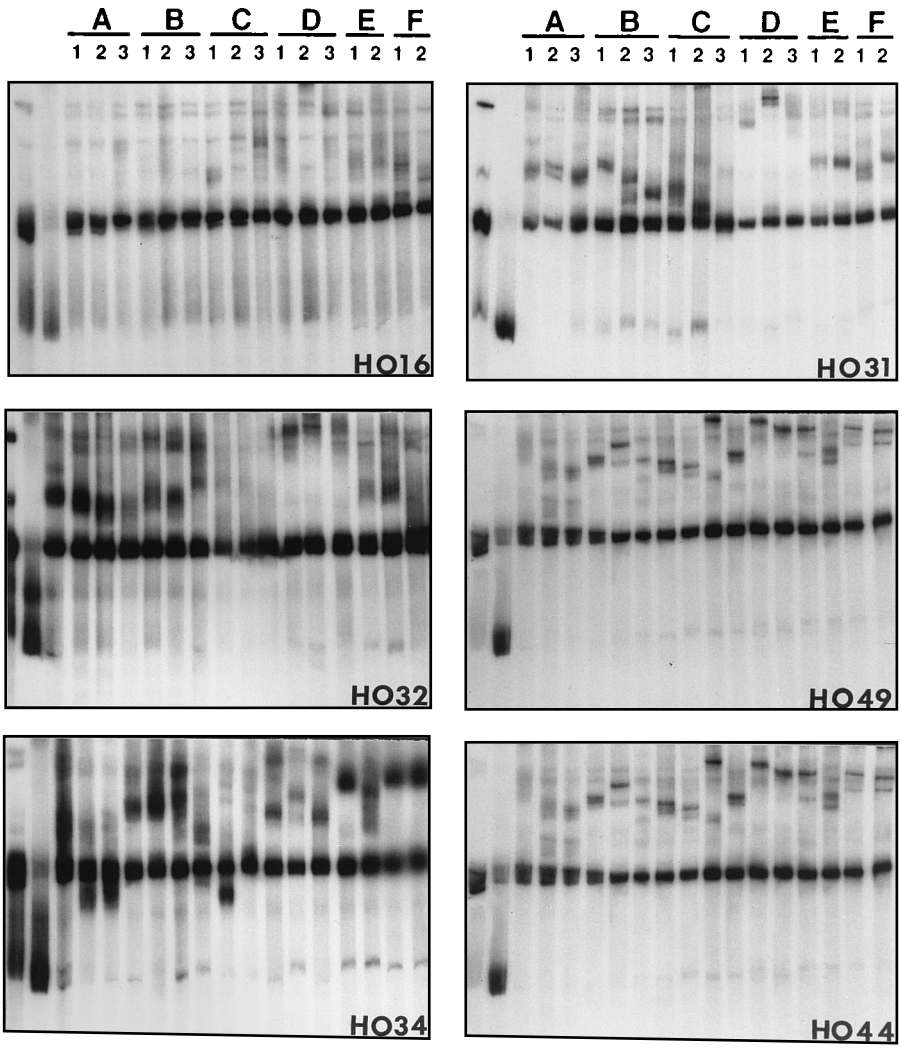

FIG. 2. Radiographs of HMA from six representative HIV-1 patients infected with HIV-1 strains of unidentified subtype (A through F). Gel descriptions are identical to those for Fig. 1. The absence of any unambiguous fast-migrating heteroduplex double-stranded DNA bands associated with reference strains of any known subtype reveals that these patients are infected with HIV-1 strains belonging to either a new subtype(s) or divergent variants within the known subtypes.

consistent with the epidemiological data in Table 1. The inter subtype diversities among Cypriot sequences within subtypes A, C, F, and I are relatively low since the isolates were derived from epidemiologically linked individuals (Table 1). The nucleotide distances between subtype I and the eight previously known subtypes are presented in Table 3. Sequences of subtype I are approximately equidistant from those of the other subtypes, in that the range of intersubtype genetic divergence of the subtype I and the other subtypes is 30.5 to 44.9%. In addition, the Cypriot subtype A and subtype F sequences form two distinct and divergent clusters within the A and F subtypes. At the present time, it is ambiguous whether these sequences belong to two new subtypes or are recombinants from existing subtypes; therefore, we have referred to them as A_{CY} and F_{CY}, respectively.

Predicted protein sequence analysis. Alignment of the predicted protein sequences from all Cypriot sequences is shown in Fig. 4. For comparisons, the consensus sequence from each subtype was also deduced and presented. The positions of all four cysteine residues in this region were highly conserved, suggesting a similar folding of this region of the Env glycoprotein. All sequences had a putative N-linked glycosylation site at the N terminus of the V3 loop. Two sequences, HO32-1 (subtype I) and HO49-1 (subtype A), had in-frame stop codons in positions that normally encoded highly conserved tryptophan (W) residues. A similar observation, occasional in-frame covariations of tryptophan residues to stop codons, were previously reported for HIV-1 Gag protein (32) and simian immunodeficiency virus Env glycoprotein (28), and it may be associated with the relative high G-to-A nucleotide transition rate observed in HIV and simian immunodeficiency virus (25) or may be an artifact during PCR amplification.

Eleven of fifteen subtype B sequences had the GPGR tetrapeptide motif at the crest of the V3 loop, which is the most common motif found throughout subtype B sequences. However, in one subtype B sequence, HO11-1, the proline (P) in the IGPGR motif was replaced by isoleucine (I), accompanied by a replacement of I by phenylalanine (F), another nonpolar residue, leading to the motif FGIGR. Similar dual replacements of residues at the tip of the V3 loop, P to I and I to a nonpolar residue, were also reported in some subtype B sequences isolated in Brazil (45, 56). The two subtype C se-

TABLE 2. Intrasubtype nucleotide diversity among HIV-1 isolates from patients in Cyprus[a]

HIV-1 subtype (*n*)	% Median nucleotide diversity (range)
A (5)	13.1 (2.4–17.6)
B (15)	15.6 (0.4–25.6)
C (2)	0.4 (NA[b])
F (2)	10.7 (NA)
I (3)	5.5 (2.0–7.5)

[a] Nucleotide distances were calculated for 27 HIV-1 sequences by the Kimura method.
[b] NA, not applicable.

quences and one subtype B sequence, HO40-1, lack the potential N-linked glycosylation site immediately after the V3 loop; this is a common feature of subtype C sequences. The tetrapeptide motifs at the tip of the loops of the five subtype A sequences were highly variable, containing two GPGQ, one EPEQ, one GPVY, and one RPRQ (contains an in-frame stop codon). It is notable that replacement of glycine (G) by arginine (R) in the tetrapeptide motif in both of the defective sequences (HO49-1, subtype A; and HO32-1, subtype I) was always accompanied by a reversion of W to a stop codon immediately preceding the V3 loop. The two subtype F sequences had the GPGQTFF motif, while the two nondefective subtype I sequences had GPGQTW and GPVQTW motifs.

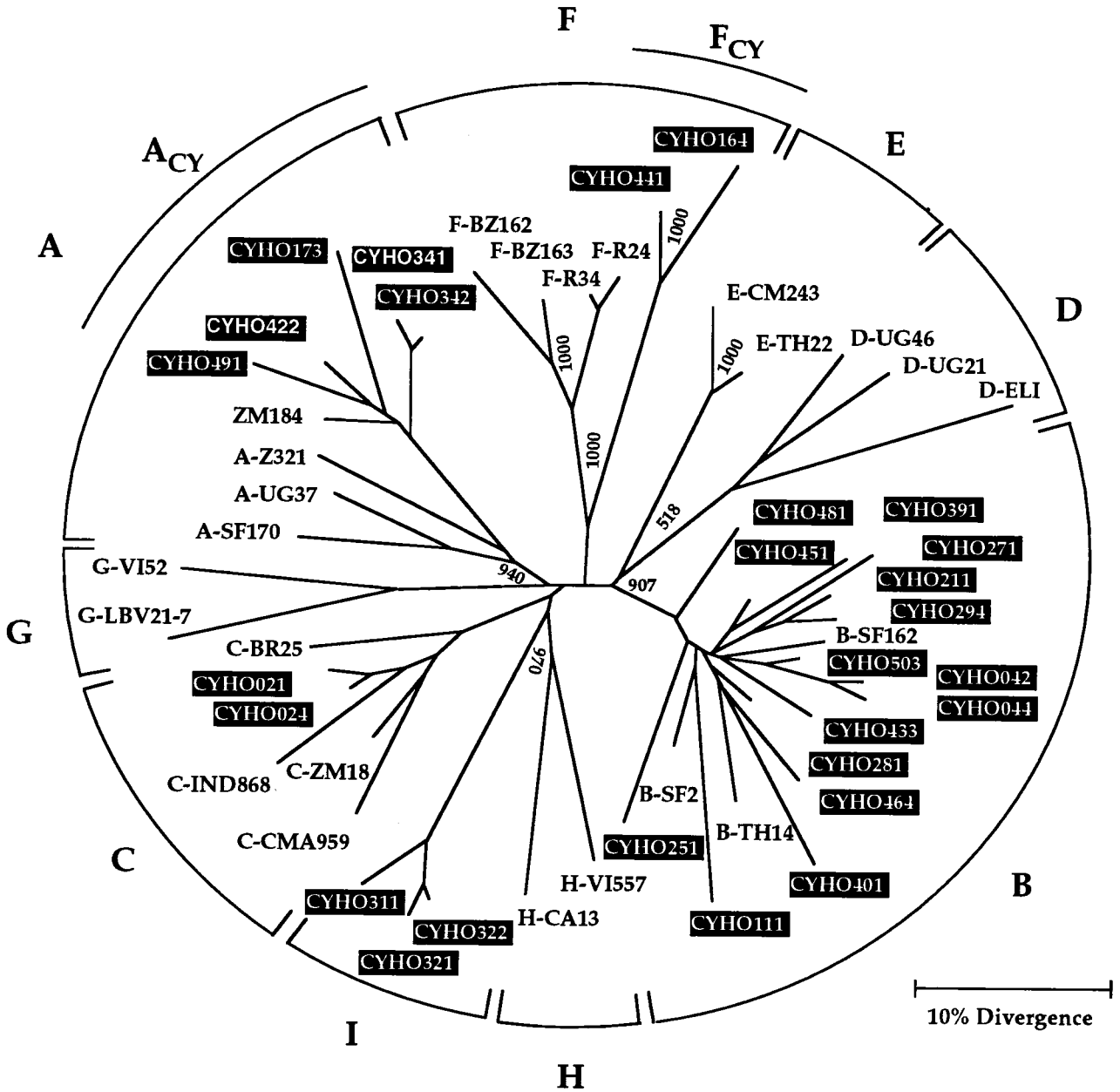

FIG. 3. Phylogenetic tree for 27 HIV-1 gp120 sequences from patients in Cyprus and 23 representative reference sequences from eight known subtypes (A through F). The sequences determined in the study are shown in white on black boxes. The divergence between any two sequences is obtained by summing the branch length, using the scale at the lower right. The numbers indicated at several nodes are consensus bootstrap values out of 1,000 replications (15). The circular brackets on the periphery of the tree denote the determined subtypes as described in Results.

TABLE 3. Intersubtype nucleotide distances between HIV-1 subtype I and previously known subtypes[a]

% Median nucleotide diversity (range)							
A[b]	B	C	D	E	F[c]	G	H
32.4 (27.2–40.1)	33.5 (28.8–42.1)	30.5 (28.6–34.8)	44.9 (37.9–49.8)	34.0 (32.0–36.2)	34.6 (31.2–42.6)	39.7 (36.8–42.3)	30.9 (29.7–32.3)

[a] Genetic distances were calculated by the Kimura method (27), and phylogenetic relationships were determined by the neighbor-joining method. The limits of the gp120 region examined were dictated by the available H reference sequences, H-CA13 and H-VI557.

[b] Median (range) nucleotide diversity between subtypes I and A_{CY} (CYHO341, CYHO342, CYHO422, CYHO491, and CYHO173) is 33.3 (27.3 to 40.1), that between I and A_{REF} (subtype A reference strains A-SF170, A-UG37, and A-Z321) is 30.8 (27.2 to 33.9), and that between A_{CY} and A_{REF} is 26.8 (19.5 to 30.8).

[c] Median (range) nucleotide diversity between subtypes I and F_{CY} (CYHO164 and CYHO441) is 38.9 (37.2 to 42.6), that between I and F_{REF} (F reference strains F-R24, F-R34, F-BZ162, and F-BZ163) is 32.4 (29.9 to 36.2), and that between F_{CY} and F_{REF} is 31.1 (29.4 to 32.7).

DISCUSSION

Among several thousand HIV-1 sequences obtained from many international sites thus far, two main phylogenetic clusters, termed M and O, have been observed (29). The vast majority of international isolates belong in group M, and they are grouped into eight subtypes designated A through H according to previously established criteria (29, 40, 50). In this study, we report the results of genetic characterization and molecular phylogeny of HIV-1 isolates from 24 patients from Cyprus, using HMA and DNA sequencing. Both methods indicated that the molecular epidemiology of HIV-1 infection in Cyprus is complex and identified the presence of a new subtype (I).

HIV-1 isolates from 24 subjects in Cyprus were classified as follows: 15 in subtype B, one in subtype C, two in subtype F, four in subtype A, and two in a new subtype which we have designated I. One subject was HIV negative by PCR but was clearly HIV-1 seropositive. There is a larger variation among isolates in subtype B compared with isolates from the other subtypes, suggesting multiple introductions of subtype B and single introductions of subtypes A, C, F, and I into Cyprus. Analysis of the HIV-1 molecular phylogeny in Cyprus is consistent with the epidemiological information from the study subjects. Cypriot subtype B sequences are genetically similar to those found in the United States and in Europe. Patients infected with subtype B strains reported traveling to or living in Europe or the United States or reported that their sexual partners had done so. Subtype C sequences isolated from one patient (HO02) who reported living in Zambia are similar to subtype C sequences found in Zambia (35).

The phylogenetic tree shows that five sequences from four patients, a three-member family (HO34, father; HO17, mother; and HO49, child) and a father's heterosexual partner (HO42), cluster in a distinct deep branch within subtype A, designated in this report A_{CY} (Fig. 3). It is noted that these sequences cluster with ZAM184; this isolate was recently identified to have a complex recombinant *env* gene, containing regions belonging to subtypes A and C together with a small

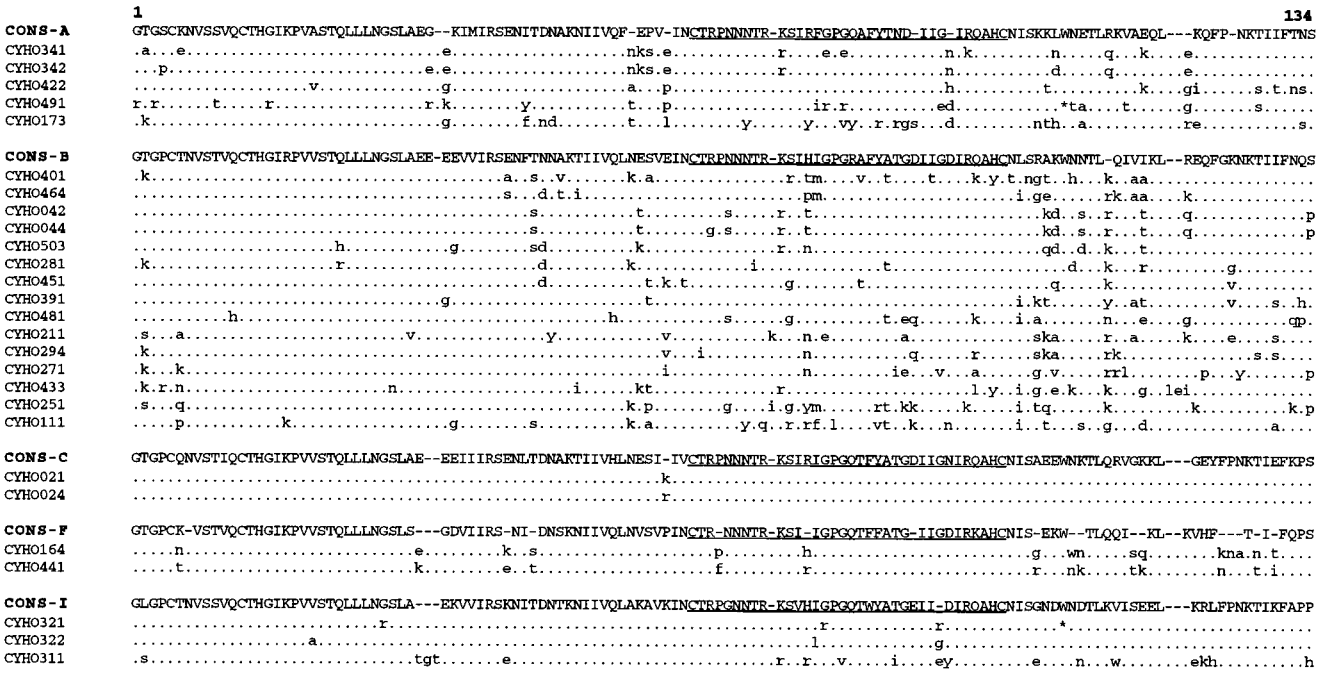

FIG. 4. Amino acid sequence alignment of the C2 to V3 region of HIV-1 *env* genes among HIV-1 isolates from patients in Cyprus. Sequence designation contains seven characters; e.g., in CYHO322, the first two characters, CY, designate Cyprus, the country of origin, the next four characters, HO32, designate the study subject's number, and the last character, 2, designates the sequence number. Consensus sequences for each of the five subtypes were deduced on the basis of the most common amino acid in a given aligned position within each subtype. The V3 region is underlined in all consensus sequences. Dots represent identity with the consensus sequences in each subtype, dashes represent gaps introduced to optimize the alignment, and asterisks (CYHO321 and CYHO491) indicate in-frame protein stop codons.

region that cannot be designated to any currently known subtype (46). Our current interpretation is either that the A_{CY} isolates belong in a new subtype in which is also included the parental virus for the unknown region found in ZAM184 or else that the A_{CY} isolates are themselves complex recombinants similar to ZAM184. If the latter explanation is true, we offer two interpretations. (i) A_{CY} is epidemiologically linked with ZAM184 or (ii) A_{CY} is a result of an independent in vivo recombination within an individual coinfected with multiple HIV-1 strains belonging to subtypes similar to those involved in the ZAM184 recombination event. The infected members of the family reported living in the United Kingdom, although the exact origin of the infecting strain and the extent of its spread in Cyprus is not known.

After completion of this study and submission of the manuscript, we found that a number of heterosexual partners of subjects HO34 and HO42, and a number of subsequent sexual partners, were infected by the same HIV-1 strain. All of the more recently infected individuals, like the four subjects described in this study, live within a 25-mile (ca. 40-km) radius on the southeast coast of Cyprus. The clinical status of and epidemiological information on the new subjects, as well as the biological and genetic properties of the new viral isolates, are currently under investigation.

Two sequences isolated from two sexual partners (HO16 and HO44), designated in this report F_{CY}, are related to subtype F sequences but form a distinct cluster which is highly divergent from subtype F sequences from Brazil (36), Romania (14), and Cameroon (42). Thus, it is not yet clear whether these sequences should be grouped into a new subtype or maintained in subtype F.

Finally, three sequences from two patients formed a distinct cluster similar to those formed by the other known subtypes. On the basis of the branch topology, the lengths of the phylogenetic tree, and the absence of knowledge of any other candidate, we propose that these new variants be designated subtype I. This subtype designation was approved by the Los Alamos National Laboratory after an independent analysis (38). The two patients infected with this new strain are heterosexual partners and former intravenous drug users living for several years in Athens, Greece. Efforts to identify more isolates within subtype I in the intravenous drug user communities in both Athens and Cyprus are now in progress in an attempt to determine the source of this new HIV-1 subtype.

In conclusion, we found HIV-1 variants in Cyprus belonging in at least five subtypes, including a new subtype designated I. This extreme diversity of HIV-1 in Cyprus is paradoxically high considering that the total population of the island is less than a million. This diversity, however, may be explained in part by the high population flow in Cyprus from many countries (12.6 million people visited Cyprus from 1983 to 1993, with an average length of stay of 11.7 days) and by the high number of temporarily expatriot Cypriots who return to Cyprus and reside there permanently.

ACKNOWLEDGMENTS

We thank all participating subjects from the Nicosia General Hospital AIDS Clinic, A. Achilleoudis of the Cyprus Association of Cancer Patients and Friends, E. Lazayou and the Cyprus Ministry of Health for valuable assistance; E. Delwart, J. Mullins, J. Levy, C. Cheng-Mayer, and the WHO Network for HIV-1 Isolation and Characterisation for providing HIV-1 gp120 clones of subtypes A to F; D. G. Pierides and T. Zhu for experimental assistance; J. Kyriannis for computer assistance; K. McInnes and G. Myers for assistance in phylogenetic analysis; A. Papageorgiou for providing information from The Cyprus Tourist Organization; W. Chen for data preparation; and E. Delwart, R. Connor, and J. Moore for helpful discussions.

L. G. Kostrikis is a former Pediatric AIDS Foundation Scholar and an Aaron Diamond Foundation Fellow. This work was supported by a Pediatric AIDS Foundation Scholar Award (77238-15-PF), an Aaron Diamond Foundation Fellowship Award (HRI 817-5332E), and grants from NIH (AI25541, AI32427, AI27665, AI35522, AI45218, AI35168, and AI24030) awarded to D. D. Ho.

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