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Genetic Analysis of Human Immunodeficiency Virus Type 1 and 2 (HIV-1 and HIV-2) Mixed Infections in India Reveals a Recent Spread of HIV-1 and HIV-2 from a Single Ancestor for Each of These Viruses

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DNA sequences encoding the surface envelope glycoproteins of human immunodeficiency virus type 1 (HIV-1) and type 2 (HIV-2) were amplified by PCR from uncultured peripheral blood mononuclear cells obtained from patients with serologically defined HIV-1/HIV-2 mixed infections from Bombay, India. HIV-1-specific PCR products were obtained in seven of seven randomly chosen doubly reactive cases, while HIV-2-specific sequences were detected in five of seven cases (71%). DNA sequence analysis showed that the HIV-1 gp120 coding sequences were closely related to each other (nucleotide sequence divergence of between 3.1 and 6.8%). Phylogenetic tree analysis placed the Indian strains within the C subtype of HIV-1, being most similar to sequences previously found in East and South Africa. The HIV-2 sequences were also closely related to each other, with an overall sequence divergence of between 5.6 and 10.5%. The low level of nucleotide divergence among Indian HIV-1 and HIV-2 sequences suggests a fairly recent introduction of each virus into this population from a single point of entry in each case. The HIV-2 sequences reported here represent the first analysis of Asian HIV-2 strains and confirm the serological pattern previously detected in India. These data show that a substantial spread of HIV-2, together with HIV-1, has appeared outside Africa in a population hitherto unexposed to HIV. These findings imply that further spread of HIV-2 worldwide is to be expected and have important implications for future vaccine and therapy development.

Since 1990, we have been screening blood samples obtained from high-risk groups, predominantly female prostitutes and their clients, attending the STD (sexually transmitted diseases) Clinic and the Godulkas Tejpal Hospital in Bombay, India. From a total of 553 positive sera, 439 (79.3%) were found to be reactive against human immunodeficiency virus type (HIV-1), 23 (4.2%) were HIV-2 positive, and 91 (16.5%) were found to be reactive against both HIV-1 and HIV-2 (39). A high proportion of HIV-2-positive sera (33% of all HIV-positive sera) has also been detected in Goa (39).

Most of the known HIV-2 and mixed HIV-1/HIV-2 infections have been found in Africa (12, 14, 31, 35, 36); it was therefore unexpected to find such a high seroprevalence of HIV-2 and HIV-1/HIV-2 mixed infections in a country of Asia. This continent was considered to be free of HIV-2 until 1990 (40).

Dually reactive sera could be the result of infection with one or the other virus accompanied by the development of cross-reacting antibodies, infection with both viruses, or infection with a recombinant virus containing epitopes for both HIV-1 and HIV-2. Several studies have shown the presence of both HIV-1 and HIV-2 sequences by PCR in samples with mixed serology (14, 31, 33, 35). In a few cases, growth of both HIV-1 and HIV-2 has been achieved after coculture of patient

peripheral blood mononuclear cells (PBMC) with donor PBMC, establishing the presence of replication-competent viruses of both types in the original sample (12, 35).

In the present study, we analyzed primary uncultured PBMC from seven dually seropositive Indian patients for the presence of both viruses by PCR with nested primer pairs specific for either HIV-1 or HIV-2. Five of the seven serologically defined mixed infections were found to contain both HIV-1 and HIV-2 sequences. DNA sequence and phylogenetic tree analysis revealed the existence of a closely related group of viruses in both HIV-1 and HIV-2 populations, consistent with a recent radiation of both types of viruses from a common source of entry into the study population.

MATERIALS AND METHODS

Patients. Blood samples were collected between January 1991 and September 1992 from patients of the STD Clinic and Godulkas Tejpal Hospital in Bombay. A detailed description of patient status and of the serological tests used has been presented elsewhere (32, 39). Most patients were classified as stage I according to the World Health Organization classification system for the HIV infection (46). All patients whose PBMC were analyzed in this study were born in India, and none reported travelling abroad.

PCR amplification and cloning. HIV-1 and HIV-2 sequences were amplified by nested PCR from 1 µg of genomic DNA obtained from uncultured PBMC. Primer pairs were designed according to the consensus sequences for HIV-1 and

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HIV-2 (26) and numbered according to HIV-1_{LAI} (1) and HIV-2_{ROD} (16) sequences, respectively. For HIV-1, the outer primers were (5' to 3'; nonhomologous nucleotides are shown in lowercase) H1e5553 (agacgatcgtcgaCCTATGGCAGGAA GAAGCGGAGAC) and H1e9266 (gacacgatcgtcGACTGAG GGATCTCTAGTTACCAGAGTC). The inner primers were H1e5792 (gacacgatcgtcgaGCAGAAAGACAGTGGCAATG) and H1e7434 (gacacgatcggatccGTCTGAGCGGTACCGTCA GCG). DNA samples from patients D744, D868, and D766 were amplified by using a different set of primers (8). For HIV-2, the outer primers were H2e6054 (5'-GGGGCTCGG GATATGGTATG-3') and H2e8416 (5'-CAAGAGGCGTAT CAGCTGGCGGATCAGGAA-3') and inner primers were H2e6128 (5'-acgatcgaattCCTGCATCAGACAAGTGAGT-3') and H2e7691 (5'-GTCGCGAGAAAACCCAAGAATTCTA GC-3').

Reaction conditions were 5 pmol of each primer, 0.2 mM deoxynucleoside triphosphates, 1.8 mM MgCl₂, and 1.25 U of *Taq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.) in 50 µl of buffer containing 10 mM Tris-HCl (pH 8.4), 50 mM KCl, and 0.01% gelatin. The thermocycling conditions were 94°C for 45 s, 55°C for 1 min, and 72°C for either 5 min (outer primers) or 2 min (inner primers). The PCR product obtained, corresponding to most of the surface (SU) protein, was cloned into the PCRII cloning vector (Invitrogen, San Diego, Calif.).

Sequence analyses and phylogenetic trees. Sequence reactions were performed by PCR cycle sequencing, using fluorescent terminators (Applied Biosystems, Foster City, Calif.) as recommended by the manufacturer. Sequence data were collected with an Applied Biosystems 373A DNA sequencer. Sequences were aligned by using the Clustal V program (17), and the resulting alignment was improved by hand editing. Alignment of the envelope sequences required the insertion of a large number of gaps. Since there is no consensus on the correct interpretation of such gaps in phylogenetic studies, and as we found that the results of our analysis were broadly similar whether or not gaps were included, all of the positions in the alignment where gaps were found were given zero weight. The synonymous and nonsynonymous changes and pairwise distances between pairs of sequences were calculated by the method of Nei and Gojobori (28). Pairwise distance matrices were also calculated by the Jukes and Cantor (18), Felsenstein maximum-likelihood (13), and Kimura two-parameter (19) methods. Since the conclusion from the matrices were very similar irrespective of the algorithm used, one of the simplest and most familiar, the Kimura two-parameter distance, was used to present the results shown here. Phylogenies were constructed by both maximum-likelihood and Fitch-Margoliash techniques and found to have identical branching orders. In addition, protein distance matrices based on the translated sequences were computed by the Dayhoff method for weighting amino acid differences (7) and found to yield similar results. The distance calculation and tree-building programs used were part of the PHYLIP suite of programs (13). Signature pattern analysis was performed by using the VESPA algorithm (20).

Nucleotide sequence accession numbers. The GenBank accession numbers for the sequences described here are pending.

RESULTS

Amplification of HIV-1 and HIV-2 sequences from doubly reactive samples. Genetic analysis was performed on seven samples (D744, D766, D808, D868, D869, D1024, and D1040) defined as HIV-1/HIV-2 mixed infections according to HIV-1/HIV-2 combination enzyme-linked immunosorbent assay

(combi-ELISA), Western blotting (immunoblotting), and immunofluorescence assays (39). PCR conditions capable of amplifying the entire surface envelope glycoprotein of HIV-1 (gp120) and HIV-2 (gp105) were used to obtain a large number of informative sites for phylogenetic purposes. The primers used for the amplification of HIV-1 and HIV-2 *env* sequences were specific for each virus. No cross-reaction was observed with control DNA under the conditions used (data not shown).

HIV-1-specific PCR products were detected in all of the seven samples. In contrast, in only five (D766, D808, D868, D1024, and D1040) of the seven samples were HIV-2-specific PCR products obtained. Amplification of the negative samples (D744 and D869) with primers specific for the protease and reverse transcriptase genes of HIV-2 were also negative. The classification of these samples as HIV-1/HIV-2 mixed infections thus may be attributed to cross-reactivity in the serology tests or to lack of sensitivity in the PCR. This latter possibility may well apply, since the detection limit of HIV-2 sequences in our experiments was 100 copies of a plasmid DNA which contained the *pol* and *env* genes of HIV-2D194 (21). Similar findings have been reported in studies from the Ivory Coast: positive PCR results were obtained for HIV-1 in 91% of the dually seropositive cases ($n = 34$) and in 71% for HIV-2 in the same samples (14). In another study, HIV-1 PCR was positive in 100% of 36 cases with double seroreactivity, and HIV-2-specific PCR was positive in only 33% of the same samples (31).

Genetic analysis of the Indian HIV-1 strains. DNA sequence analysis of the *env* region of five Indian HIV-1 strains (D744, D766, D808, D868, and D1024) showed these sequences to be closely related to each other (Table 1). The nucleotide divergence between these sequences, which include all variable regions of gp120, was between 3.1 and 5.8%. Moreover, when the nucleotide sequences of these strains were compared with those originating from single HIV-1 infections from Bombay (D747, D757, and D760) and Goa (D1044) (11), a high degree of sequence similarity was again observed (Table 1). The overall sequence similarity among all these Indian HIV-1 strains ranged between 3.1 and 6.8%, with a mean genetic distance of 5.7%. In contrast, the mean genetic distance outside the Indian group was 18.1% (Table 1). The Indian HIV-1 strains were most similar to sequences obtained from Zambia (HIV_{ZAM18}) and South Africa (HIV_{NOF}). Phylogenetic trees were constructed by maximum-likelihood, neighbor-joining and Fitch-Margoliash methods and found to be essentially identical. Bootstrap resampling of the data set was performed, and the final inferred relationships between samples were optimized by global branch swapping. One thousand bootstrap resamplings of the data were performed, and the frequency with which the major branch to the Indian data set was found was 91.9% (Fig. 1). The Indian HIV-1 sequences appear in the tree as a monophyletic group closely related to the HIV_{ZAM18} and HIV_{NOF} sequences. These sequences are members of the C envelope sequence subtype of HIV-1, whose members include viruses isolated from Djibouti, Somalia, Uganda, Zambia, Senegal, Zimbabwe, Malawi, and South Africa (8, 11, 27, 29).

Alignment of the deduced amino acid sequences for the Indian strains showed some remarkable characteristics (Fig. 2). The V3 loop of the Indian gp120 was relatively conserved, with an average intersample variation of 4.4% (range, 0 to 8.6%). However, the positions of amino acid changes within the V3 loop were not random. With a single exception, the amino acid sequence RIGPGQTFYATGDIIGDIR was found in all Indian strains ($n = 9$) at the loop apex. Within this region, only

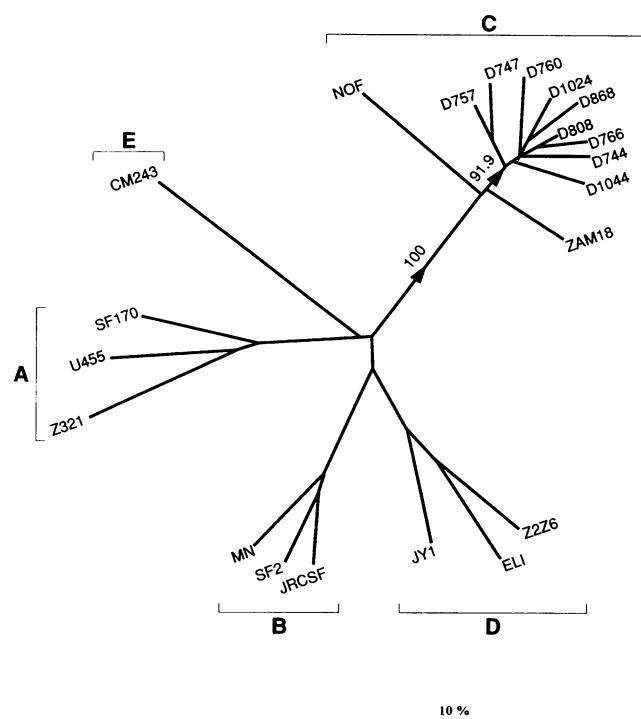


FIG. 1. Phylogenetic relationships of the V1-V5 region of HIV-1 *env* genes from the Indian strains to other HIV-1 strains. The tree was obtained by Fitch-Margoliash methods on the basis of the nucleotide distances summarized in Table 1 (see text for details). The nucleotide sequences used were obtained from the Los Alamos data base with exception for the HIV_{Zam18} sequence, which was kindly provided by Joost Louwagie prior to publication. The degree to which the data support the Indian branch is indicated by a value denoting the number of times (percentage) such a branch appears in 1,000 trees generated by bootstrap resampling of the data.

25% (one of four) of the nucleotide substitutions changed the amino acid sequence, whereas 66% of the nucleotide changes over the remainder of the V3 loop led to changes in the amino acid sequence. These results indicate that these 19 conserved amino acid are under considerable selective constraints, while sequences surrounding this motif are evolving rapidly.

All Indian gp120s lack a potential N-linked glycosylation site at the 5' end of the V3 loop (position 197 in Fig. 2), a property shared among all C-subtype sequences which is otherwise present in the vast majority of the HIV-1 sequences analyzed to date (11, 27). The lack of this glycosylation site has been found in some but not all cases of perinatal transmission of HIV (29, 45). An N-linked glycosylation site not found in most HIV-1 sequences is present in most C-subtype sequences, close to the C terminus of the CD4 binding domain (position 345 in Fig. 2) (11).

Genetic analysis of the Indian HIV-2 strains. Nucleotide sequence analysis of the HIV-2 *env* clones obtained from four of the seven dually reactive samples from Bombay (D1024, D766, D808, and D868) revealed a close intersample relatedness (mean genetic distance, 7.3%; range, 5.6 to 9.0%; Table 2), similar to that described above for HIV-1. In parallel, the HIV-2 sequence from a dually reactive sample from a female prostitute living in the state of Goa (patient D1071) was found to be closely related to the Bombay sequences, with an overall sequence divergence of between 7.0 and 10.5% (Table 2),

TABLE 1. Nucleotide distances in the V1-V5 region of HIV-1 among various HIV-1 isolates^a

	D744	D747	D757	D760	D766	D808	D868	D1024	D1044	ZAM18	NOF	SF170	Z321	U455	MN	SF2	JRCSF	JY1	ELI	Z226
D744																				
D747	0.0689																			
D757	0.0585	0.385																		
D760	0.0619	0.0678	0.0654																	
D766	0.0528	0.0664	0.0628	0.0596																
D808	0.0439	0.0598	0.0518	0.0463	0.0308															
D868	0.0583	0.0768	0.0630	0.0619	0.0583	0.0472														
D1024	0.0516	0.0688	0.0573	0.0551	0.0527	0.0416	0.0439													
D1044	0.0595	0.0677	0.0608	0.0630	0.0605	0.0461	0.0618	0.0572												
ZAM18	0.0734	0.0841	0.0805	0.0850	0.0745	0.0723	0.0827	0.0791	0.0779											
NOF	0.1094	0.1026	0.0998	0.1132	0.1033	0.0986	0.1081	0.1093	0.1078	0.1053										
SF170	0.2071	0.1960	0.1926	0.2088	0.2015	0.1964	0.2085	0.2135	0.2018	0.1917	0.2200									
Z321	0.2457	0.2275	0.2256	0.2323	0.2278	0.2295	0.2319	0.2431	0.2407	0.2300	0.2530	0.1140								
U455	0.2178	0.2110	0.2031	0.2285	0.2069	0.2032	0.2154	0.2164	0.2185	0.1997	0.2205	0.1054	0.1133							
MN	0.2199	0.2155	0.2088	0.2134	0.2113	0.2086	0.2195	0.2120	0.2044	0.1965	0.2159	0.1843	0.2018	0.2004						
JRCSF	0.2129	0.2071	0.2009	0.2105	0.2125	0.2078	0.2166	0.2112	0.2044	0.1986	0.2193	0.1821	0.1910	0.2046	0.0732	0.0607				
JY1	0.2004	0.1990	0.1942	0.2011	0.2041	0.1908	0.2028	0.1982	0.1961	0.1792	0.2112	0.1694	0.1993	0.1864	0.1616	0.1584	0.1605			
ELI	0.2158	0.2161	0.2068	0.2120	0.2147	0.2016	0.2151	0.2161	0.2096	0.1987	0.2385	0.1979	0.2170	0.2030	0.1602	0.1703	0.1698	0.1141		
Z226	0.2223	0.2110	0.2071	0.2230	0.2219	0.2069	0.2147	0.2144	0.2096	0.2074	0.2333	0.1864	0.2044	0.1885	0.1592	0.1596	0.1711	0.0991	0.0888	
CM243	0.2196	0.2172	0.2123	0.2296	0.2148	0.2111	0.2237	0.2203	0.2138	0.2052	0.2199	0.1826	0.2038	0.1912	0.1946	0.1868	0.1891	0.1875	0.2174	0.2140

^a The weighted pairwise genetic distances were calculated by the Kimura two-parameter method. Gaps in the alignment were ignored in the calculation. Mean distance within the Indian group = 0.0569, $\sigma_n - 1 = 0.0098$, $n = 36$. Mean distance outside the Indian group = 0.1812, $\sigma_n - 1 = 0.0426$, $n = 66$.

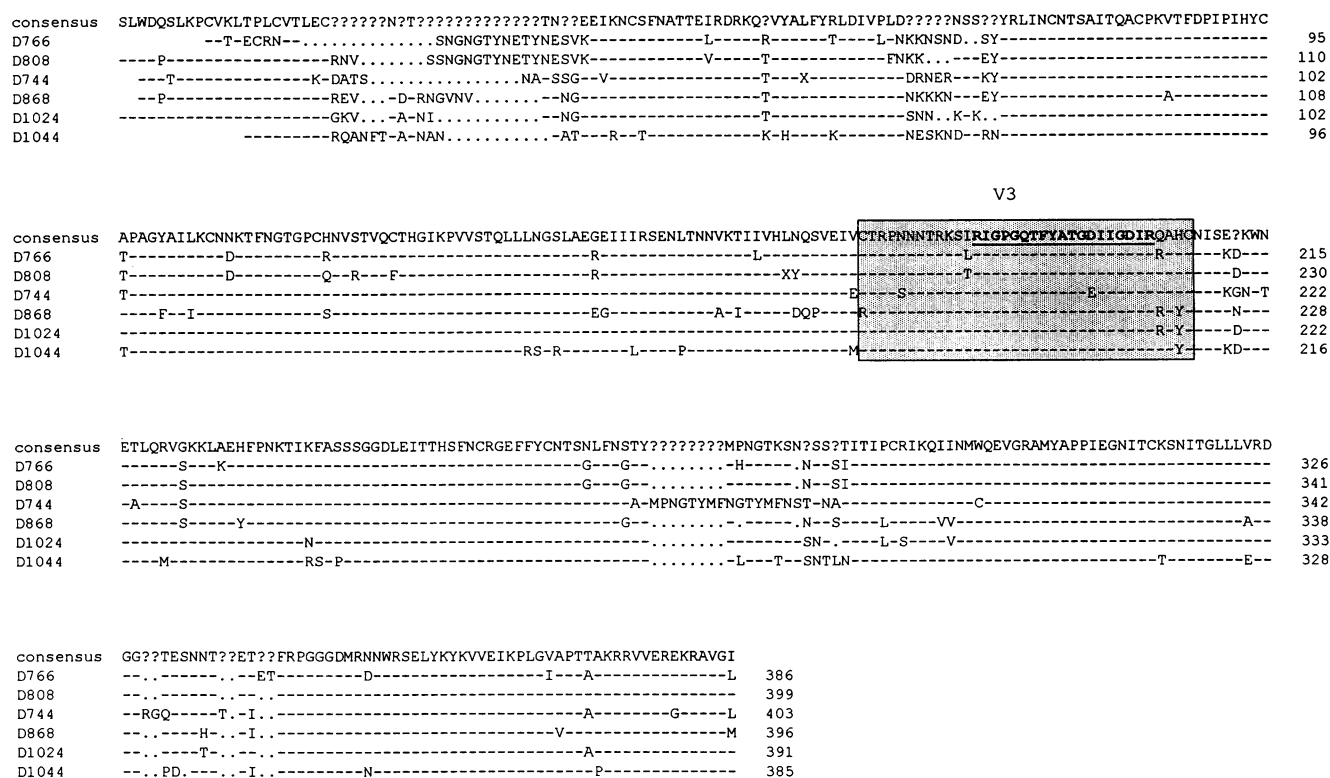


FIG. 2. Amino acid sequence alignment among the Indian HIV-1 strains. The consensus sequence was deduced from the most common amino acid at each position in the alignment and includes all Indian HIV-1 amino acid sequences ($n = 9$) available to date (reference 11 and this work). The V3 region is marked as a grey box. The 19 conserved amino acids at the V3 loop apex are shown in boldface within the V3 loop.

suggesting that the HIV-2 strains found in the Bombay cohort can also be found elsewhere in India. Interestingly, this patient had been HIV-2 positive only in April 1992 but was found to have seroconverted to double seroreactivity in July of the same year.

The mean genetic distance among all Indian HIV-2 sequences was 7.7% (range, 5.6 to 10.5%), while the mean genetic distance outside the Indian group was 23% (Table 2). To ascertain how this variation range related to the degree of diversity of HIV-2 genomes within one patient, the inpatient sequence diversity was analyzed in patient D1044. Three HIV-2 clones obtained from patient D1044 were sequenced over the C2-V3 region (346 bp; corresponding to positions 6820 to 7166 of HIV2_{ROD}). The inpatient nucleotide sequence diversity for patient D1044 was calculated to be 3.6% (range, 2 to 5%; data not shown) and thus lies within the range of what is known for HIV-1 after several years of infection (22, 25). Within the same region, the mean interpatient nucleotide sequence divergence between all five Indian HIV-2 strains was 7.5% (range, 4 to 10%), while the interpatient variation range, estimated from a reference set of sequences ($n = 9$) (27), was between 7 and 34%. Thus, the range of sequence variation between the Indian HIV-2 samples lies between the inpatient and typical interpatient diversity range, an observation previously reported for samples obtained in transmission studies (2, 4, 15, 30).

Phylogenetic tree analysis, performed as described above for HIV-1, placed the Indian HIV-2 sequences in a monophyletic cluster with the closest similarity to HIV-2_{ROD} (Fig. 3). The Indian branch was found within the A HIV-2 subtype in 100% of the trees generated by 1,000 bootstrap resamplings of the

data, as opposed to the alternative subtype B (10, 27). The deduced amino acid sequences of the Indian HIV-2 strains were found to be closely related to each other, again with a notable conservation of amino acids in the V3 region. Of the 34 amino acids within the V3 loop, 24 were common to all Indian strains, including Goa isolate D1071. The intersample genetic variation in this region between the Indian samples was between 0.0 and 5.8%, while the range of intersample variation outside the Indian group was between 11 and 16% (3). Overall, the greatest diversity was found at the N terminus of the region analyzed within the variable regions V1 and V2 and at the 3' end of the V3 region. As with the Indian HIV-1 strains, a notable conservation of amino acids was apparent in the Indian HIV-2 sequences. Since these common amino acids may reflect sites of functional significance, we investigated whether they were absent or rare in other HIV-2 sequences. For this, the VESPA algorithm (20) was used. This algorithm searches for positions within an amino acid alignment at which all five Indian sequences differ from what is predominantly found in a reference set of sequences. The reference set included all HIV-2 amino acid sequences ($n = 20$) available from the HIV sequence data base (27). From 153 amino acid sites analyzed within the C2-V3 region (positions 119 to 272 in Fig. 4), six noncontiguous amino acids were found to be shared by all or the majority of the Indian sequences and absent or rare at the same positions in the reference set of sequences (Table 3). The deduced Indian pattern was Y, A, S, K, S, and A (positions 119, 142, 173, 174, 189, and 270), while the most common amino acids at these positions in the reference set were F, T, G, R, T, and V. The frequency with which the Indian pattern of amino acids was present in the Indian HIV-2 sequences ranged

TABLE 2. Nucleotide distances in the V1-V4 region of HIV-2 among various HIV-2 isolates^a

	D1024	D766	D808	D868	D1071	CAM2	GH1	ST	ROD	ISY	NIHZ	BEN	D194	SIVMM251	D205
D1024															
D766	0.0707														
D808	0.0678	0.0899													
D868	0.0557	0.0831	0.0719												
D1071	0.0693	0.1046	0.0735	0.086											
CAM2	0.1335	0.1729	0.1533	0.1519	0.1602										
GH1	0.1279	0.1570	0.1426	0.1366	0.1444	0.1269									
ST	0.1366	0.1801	0.1544	0.1473	0.1522	0.1507	0.0944								
ROD	0.1294	0.1573	0.1394	0.1339	0.1431	0.1277	0.1190	0.1278							
ISY	0.1541	0.1938	0.1755	0.1741	0.1684	0.1581	0.1338	0.1366	0.1560						
NIHZ	0.1483	0.1849	0.1681	0.1559	0.1675	0.1231	0.1352	0.1437	0.1304	0.1473					
BEN	0.1916	0.2109	0.2067	0.1933	0.2047	0.2003	0.1514	0.1809	0.1844	0.1908	0.1880				
D194	0.1872	0.2160	0.1970	0.1956	0.2073	0.1570	0.1460	0.1566	0.1686	0.1738	0.1637	0.1030			
SIVMM251	0.3030	0.3358	0.3302	0.3187	0.3209	0.3093	0.2993	0.2900	0.3066	0.3214	0.3006	0.2969	0.2930		
D205	0.3385	0.3554	0.3488	0.3389	0.3566	0.3310	0.3051	0.3209	0.3336	0.3367	0.3367	0.3322	0.3337	0.3613	
UC1	0.3477	0.3779	0.3623	0.3484	0.3640	0.3284	0.3103	0.3219	0.3333	0.3419	0.3378	0.3473	0.3299	0.3331	0.2018

^a The weighted pairwise genetic distances between HIV-2 isolates were calculated by the Kimura two-parameter method. Gaps in the alignment were ignored in the calculation. Mean distance within the Indian group = 0.07726, $\sigma_{n-1} = 0.0138$, $n = 10$. Mean distance outside the Indian group = 0.22967, $\sigma_{n-1} = 0.0888$, $n = 66$. Mean distance outside the Indian group (ignoring last three) = 0.15341, $\sigma_{n-1} = 0.0269$, $n = 36$.

between 80 and 100%. In contrast, these amino acids were found at the corresponding positions in less than 35% of the reference set.

DISCUSSION

The genetic analysis described in this work confirms the existence of HIV-1/HIV-2 dual infections in the Bombay cohort, a situation previously suggested on the basis of serological data (39). Of the serologically defined dual infections analyzed in this study, five of seven were found to contain HIV-1 and HIV-2 gene sequences. The sequential use of the HIV-1/-2 combi-ELISA, Western blots, immunofluorescence, and PeptiLav assays used in our serological studies proved to be generally reliable in providing a serotype classification of the samples. Previous studies in which genetic characterization of dually reactive samples was attempted have shown coinfection with HIV-1 and HIV-2 in 23 to 62% of the samples (12,

14, 31, 33, 35). The discordance between the serology and the PCR results may be the result of either a failure in the amplification of HIV-2 sequences caused by the low sensitivity of the HIV-2 PCR, low copy number of HIV-2 proviral DNA, high sequence heterogeneity at primer annealing sites in the HIV-2 *env* region, cross-reactivity among HIV-1 and HIV-2 in the serological tests used, or a combination of these factors.

All Indian HIV sequences were found to be closely related to each other irrespective of the material used for the amplification (primary PBMC versus tissue culture material [this work and reference 11]) or the geographical origin of the sample (Bombay versus Goa).

The close relatedness between Indian HIV-1 strains and between Indian HIV-2 strains suggests that all are recently derived from common ancestors. To test this hypothesis, a *t* test was performed to assess the mean distance within the Indian samples in comparison with the other samples used in the analysis. When this test was performed for the HIV-1 and HIV-2 data sets (Tables 1 and 2), the test values were 18.2 (100 df, $P < 0.001$) and 8.6 (44 df, $P < 0.001$), respectively (the extremely divergent samples SIVMM251, D205, and UC1, used as outgroups in constructing the phylogenetic tree, were ignored in calculating the *t* test for HIV-2). These results strongly support the conclusions from the phylogenetic tree. Other studies, both on relationships between serial samples from the same individual (41, 43, 44) and on samples from cases for which the route of transmission is known or suspected (2, 4, 15, 30, 42) have shown that pairwise genetic distances of between 0 and 10% are typically found between clones of the envelope gene within individuals and that similar distances are found between congeneric samples originating from a common source. The mean pairwise distances within the groups (5.7% for HIV-1 and 7.7% for HIV-2) from India are therefore small enough for the groups to be considered as being derived from a single common ancestor.

In contrast to HIV-1, a close clustering of HIV-2 sequences has not been described previously. Since the intersample variation among HIV-2 strains is similar to that for HIV-1 (3) and assuming a similar rate of divergence per year for HIV-1 and HIV-2, the higher degree of divergence among the HIV-2 strains (7.7% versus 4.8% for HIV-1) may imply that the HIV-2 infection preceded the onset of HIV-1 infection in this group.

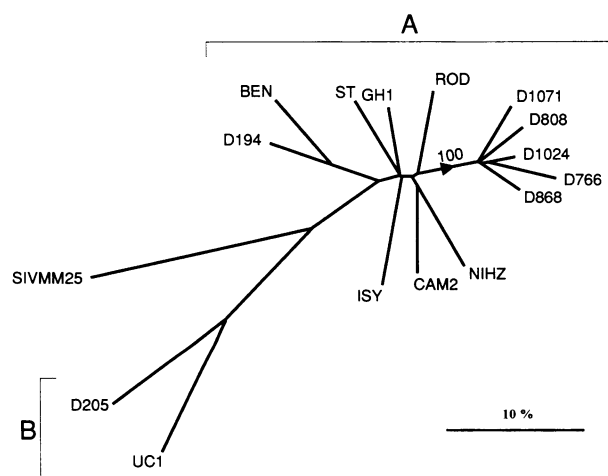


FIG. 3. Phylogenetic relationships of the V1-V4 region of HIV-2 *env* genes from the Indian strains with other HIV-2 strains. The tree was constructed from the data in Table 2, using the methods described for Fig. 1.

N.I. cons	MNCN?T?????????TTT?T??T????I?E?SPCI??DNCTGLGEEE?V?CQFNMT	
Indian cons	MNCSKNSGTST.....GNTTT?TA????S?INETS?CIRTNNCSGLREEE?VSCQF?MT	
D766	-----S---PA---I-M---P---D-----M---S--	52
D868	-----D-S-----P-S---SYV---S-H-DT-----I---S--	52
D1024	-T-R-S-----A-S-PMINET-I---P-----M---N--	56
D808	-----YN---A-----P---E-M---N-S---A-----E---I-N---N-A	52
D1071	-T-----I-----S---T---DAAMV---Q---A-----L---D--	53
N.I. cons	GLERDKKKQYNETWYSKDVVC???T.....???????CYMNHNCNTSVITESCDKHYWD	
Indian cons	GL?RDKPKRYNETWYSSDVVCE?????????NNTSNQSRCYMNHNCNTSVITESCDKHYWD	
D766	--T-----TTG.....AT-----D-----	107
D868	--A-----ATG.....T-D-L-----N-----	107
D1024	--A---G-----TE.....T-----	110
D808	--P---W-----R-----NPT-N-----E----	103
D1071	--T-----DAPTNTSNQTS---D---C-----E---X-	114
N.I. cons	AMRFRYCAPPGFALLRCNDTNYSGFEPNCSKVVASTCTRMETQTSTWFGFNGTRAENRTY	
Indian cons	DMRFRYCAPPGYALLRCNDTNYSGFEPNCSKVVASTCTRMETQTSTWFGFNGTRAENRTY	
D766	--W-----YT-----C-A---K-----	168
D868	-----S-----S-----C-A---K-----	168
D1024	-----F-----C-A---K-----	171
D808	-I---T-----K-----R-----	164
D1071	AI-----K-----R-----	175
N.I. cons	IYWHGRDNRTIISLNKYINLT?HCKRPGNKTVPITLMSG??FHSQ?P?INKRPRQAWCF	
Indian cons	IYHWSKDNRTIISLNKHYNLISYCKRPGNKTVPITLMSGVLFHSQ.P.INNRPRQAWCF	
D766	-F---G-A---C-TYD-----E-----	227
D868	V-----Y---H-----R-----	227
D1024	-----H-----R-----	230
D808	-----T-----R-----	223
D1071	-----R-----T-----S-----	234
N.I. cons	KG?W?EAMQEVKQTLAKHPRYKGTND?T??I?F?A?PG?GSDPEVAYMWTNCRGEFLYCNM	
Indian cons	KG?WKEAMQEVKQTL?KHPRYRGTN?.TGKINF?A.PGKGSDDP?AYMWTNCRGEFLYCNM	
D766	T-T-R-----V-R-----E.-R---T-----	286
D868	T-K-----R-R-IAE-----N.-N-K-V-----	286
D1024	--K-----V---E---K.-ED-H-A-----V-----	289
D808	--R-----E-T-----D.-A-----	282
D1071	--Q-----A---K---N-----T-.-S-----I-----	293
N.I. cons	TWFLNWVENKT.NQT?RNYAPCHI?QIINTWH	
Indian cons	TWFLNWIENRT?N?TWHNYVPCHIKQIINTWH	
D766	-----V-R-----	318
D868	-----K-E-A-----	318
D1024	-----M-K-----	321
D808	-----V--K-A-Q-----	314
D1071	-----XV-D--A-R-----	325

FIG. 4. Amino acid sequence alignment of the V1-V4 region of HIV-2 *env* genes from the Indian strains. The V3 loop is shown as a grey box. The amino acids defined in the Indian HIV-2 signature pattern are shown in white. Lines above the sequence delimit the antigenic epitopes previously described by de Wolf et al. (9). Only those epitopes which show polymorphism in the samples shown here are included. The non-Indian (N.I.) consensus (cons) sequence was derived from nine HIV-2 sequences available from the HIV sequence data base (27).

The rapid spread of HIV-1 and HIV-2 within a short time period in the Bombay and Goa cohorts is puzzling. Either social and behavioral factors in the population studied have led to efficient transmission of these particular genotypes or the Indian HIV strains have a particularly high potency for transmission due to some inherent properties of the virus. Changes in social and behavioral factors are difficult to assess in a short time period. However, examination of the amino acid sequences at the V3 loop of the Indian HIV-1 strains revealed the presence of an uncharged amino acid at position 11 (S or G), an acidic amino acid at position 25 (D or E), and the noncontiguous amino acids K, Y, G, I, D, and I, all of which are typical of macrophage-tropic variants (5).

A similar case of rapid spread of closely related HIV-1

genotypes has been described for predominantly heterosexual transmission in northern Thailand (24). Examination of the northern Thai sequences revealed an uncharged amino acid at position 11 (S), an acidic amino acid at position 25 (D), and the noncontiguous amino acids Y, G, I, D, and I at positions equivalent to those prevalent found in Indian and monocytotropic strains (5). Thus, it appears that the spread of HIV-1 in the west coast of India and northern Thailand might be explained by the primary introduction of a monocytotropic HIV-1 variant into high-risk groups living in these regions. These results are in agreement with previous observations which have suggested that macrophage-tropic variants predominate in primary infection (6, 37, 47).

Previous studies have suggested that HIV-2 is less patho-

TABLE 3. Definition of an amino acid sequence pattern for the Indian HIV-2 strains

HIV-2	Amino acid frequency					
	Indian HIV-2 pattern ^a					
	Y	A	S	K	S	A
Indian	0.80	1.00	1.00	0.80	1.00	1.00
Reference set ^b	0.20	0.35	0.00	0.35	0.30	0.05
	Non-Indian Pattern					
	F	T	G	R	T	V
Indian	0.20	0.00	0.00	0.20	0.00	0.00
Reference set ^b	0.75	0.35	1.00	0.60	0.65	0.95

^a Defined by those positions at which the amino acid sequences of the majority (>80%) of the Indian HIV-2 strains differ from what is predominantly found in a monophyletically related set of sequences.

^b Includes all A-subtype HIV-2 sequences ($n = 20$) available from the HIV sequence data base (27).

genic than and not transmitted as efficiently as HIV-1 (7a, 23, 24). In contrast, our results (reference 39 and this study) indicate that HIV-2 can spread rapidly within a defined population. Almost 21% of all HIV infections in Bombay were found to be HIV-2 reactive either alone or in combination with HIV-1. The existence of six amino acids in the external envelope protein which were conserved in most of the Indian HIV-2 strains and rare or absent in non-Indian strains may define these amino acids as sites of potential functional importance for the spread of the virus. Four of the six amino acids map in close proximity to, or within, regions previously identified as antigenic epitopes of the HIV-2 envelope surface protein (9). If these changes alter the antigenic properties of the Indian HIV-2 strains, they might have evolved as immunological escape mutants. Neutralization assays will be required to test this hypothesis. Alternatively, the noted conservation may be simply a reflection of the low level of sequence divergence in a young epidemic.

In summary, we have found a significant spread of HIV-2 together with HIV-1 in Bombay in a time period early in the epidemic, as shown by the low interpatient genetic divergence observed. In particular, the high incidence of HIV-2 infections is indicative of a new HIV-2 epidemic outside Africa, happening in parallel to an HIV-1 epidemic. This is the first time that this situation has been observed. In patient 1071, we have observed HIV-1 superinfection of an HIV-2-infected individual. Whether concomitant infections with both viruses occur is unknown at present. Clearly, however, the aggressive disseminated form of Kaposi sarcoma, a condition so far not observed in this country, has also reached India (patient 1024 [38]). The fact that many of the HIV-2 infections are accompanied by an HIV-1 infection may have unforeseen consequences for the pathology and further dissemination of HIV-2.

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