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Indian primary HIV-2 isolates and relationship between V3 genotype, biological phenotype and coreceptor usage

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Abstract

The chemokine coreceptors play a significant role in HIV entry and pathogenesis. The V3 region of HIV envelope glycoprotein is considered as a principal determinant for viral phenotype and tropism. The present study describes lack of association between the V3 genotype and viral phenotype of 18 Indian HIV-2 isolates. The viruses were isolated, confirmed by PCR and the HIV subtypes were determined by sequencing V3 region of the *env* gene. The coreceptor usage and syncytium inducing (SI) capacity of isolates was determined. Our study indicated that CCR5 coreceptor usage and NSI phenotype is predominant among Indian HIV-2 isolates obtained from patients in the early stage of infection. Two of the four HIV-2 isolates obtained from the late stage patients were SI and dual tropic. Phylogenetic analysis of these isolates revealed close relatedness to the isolates from western and southern India.

Keywords: HIV-2 isolates; V3 genotype; Biological phenotype; Coreceptor usage; India

Introduction

The chemokine receptors play a significant role in HIV cell tropism and pathogenesis (Philpott, 2003). HIV-1 subtype B-infected individuals in early and asymptomatic stage mostly harbor non-syncytium inducing (NSI) and CCR5 tropic (R5) viruses, while those in the late or symptomatic stage harbor syncytium inducing (SI) and CXCR4 (X4) or dual tropic (X4/R5) viruses (Connor et al., 1997), demonstrating a switch in coreceptor usage during disease progression (Connor et al., 1997). Such a coreceptor switch was not observed in the HIV-1 subtype C infections (Abebe et al., 1999; Cecilia et al., 2000; Peeters et al., 1999). It was also demonstrated that the emergence of X4 or X4/R5 and SI HIV-1 variants was a predictor of accelerated disease progression (Hayman et al., 2004), rapid decline in CD4-positive T cells (Philpott, 2003) and broadened coreceptor usage (Hoffman and Doms, 1998).

In contrast to HIV-1, HIV-2 isolates were reported to use a broad range of coreceptors irrespective of the disease stage. It was demonstrated that a majority of the HIV-2 isolates like SIV used CCR1, CCR2b, CCR3, CCR8, BOB, BONZO, CX_3CR1, gpr1, APJ and US28 coreceptors in addition to CXCR4 and CCR5 coreceptors (McKnight et al., 1998; Morner et al., 1999). The X4 viruses isolated from patients with late stage of HIV-2 infection indicate a similar switch in coreceptor usage during HIV-2 disease progression (Morner et al., 1999; Sol et al., 1997).

The phenotypic switch has been associated with mutations in the V3 region of *gp*120 envelope glycoprotein. Sequence analysis of a large panel of V3 domains of SI and NSI HIV-1 isolates has revealed that the occurrence of positively charged amino acids at positions 11 and 25 are associated with SI phenotype and also that the presence of neutral or negatively charged residues are predictive of NSI phenotype (De Jong et al., 1992; Fouchier et al., 1992). Although the V3 region of HIV-2 envelope glycoprotein has

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been shown to be involved in viral fusion (Freed and Myers, 1992), less is known about determinants of cell tropism within this region. Studies by Albert et al. (1996) and Morner et al. (1999) have found correlation between V3 genotype and biological phenotype, as measured by the net charge, sequence heterogeneity and positively charged amino acids at positions 9 and 10 in the V3 region of *gp* 105. Previous studies on most of the V3 sequences from Indian HIV-2 isolates from western and southern part of India have reported comparable amino acid sequences, but the correlation of this with phenotypic characteristics and coreceptor usage has not been studied (Grez et al., 1994; Kannangai et al., 2003).

In spite of the ability to use a broad range of coreceptors leading to increased cell range, HIV-2 is less pathogenic and less transmissible than HIV-1 (Kanki et al., 1994; Marlink et al., 1994). HIV-1 pathogenesis in context with coreceptors has been studied in detail, but the significance of broad coreceptor usage of HIV-2 in pathogenesis still remains unclear. We report here an absence of correlation between the genetic features of V3 region of *gp* 105 and viral phenotype of the isolates from Indian HIV-2-positive individuals.

Results

Profile of HIV-2-positive individuals

Among the isolates obtained from the HIV-2 seropositive individuals, 17 were from the state of Maharashtra and one from the Gujarat state in the western region of India. The mean age of participants was 34 years (range, 23–50 years), 15 were males and three were females. Twelve individuals had acquired infection through heterosexual route. One individual had a history of multiple injections in clinical settings. There was no reported risk factor for HIV infection in five individuals.

Classification of the HIV-2-positive individuals

The absolute CD4 count could be determined in 14 out of 18 individuals. The median absolute CD4 count was 283 cells/mm^3 (range, 78–830 cells/mm^3). The individuals were classified based on the clinical presentation and CD4 counts as per CDC AIDS Surveillance case definition—1993 (CDC, 1992). The CDC categories A1, A2, B1 and B2 were classified as early HIV disease and A3, B3, C1, C2 and C3 were classified as advanced HIV disease. Of 14 individuals, nine were in the early stage of the disease and four in the late stage (Table 1). The median CD4 count of individuals in the early stage of disease was 327 cells/mm^3 (range, 227–755 cells/mm^3) and that in the late stage was 95 cells/mm^3 (range, 78–401 cells/mm^3). In addition, one individual presented with pulmonary tuberculosis but the CD4 count of this individual was normal (830 cells/mm^3). In view of the normal CD4 count, this individual could not be classified as having late stage HIV disease. The CD4 count was not available for four individuals of whom, two were asymptomatic and two had pulmonary tuberculosis.

Coreceptor usage of HIV-2 isolates

The GHOST cells expressing CXCR4 or CCR5 coreceptors were used for determining coreceptor usage. Out of 18 HIV-2 isolates, nine isolates from patients in the early stage of disease used CCR5 coreceptor, however, titres of two isolates (Fig. 1, NARI-5 and NARI-7) were low. Two of

Table 1
Phenotypic characteristics and V3 amino acid sequence of HIV-2 isolates

Isolate no.	Disease stage (CDC, 1993)	Coreceptor usage	Syncytium induction (MT2)	V3 sequence	Net charge
NARI-1	A1	R5	NSI	CKRPGNKTVVPITLMSGLVFHSQPINNRPRQAWC 34	6
NARI-2	A1	R5	NSI	CKRPGNRTVVPITLMSGLVFHSQPINTRPKQAWC 34	6
NARI-3	A2	R5	NSI	CKRPGNKTVVPITLMSGLIFHSQPINNRPKQAWC 34	6
NARI-4	A2	R5	NSI	CKRPGNRTVVPITLMSGLVFHSQPINTRPKQCWC 34	6
NARI-5	A2	R5	NSI	CKRPGNRTVVPITLMSGLVFHSQPINTRPKQC 32	6
NARI-6	A2	R5	NSI	CRRPGNKTVVPITLMSGLVFHSQPINDRPRQAWC 34	5
NARI-7	A2	R5	NSI	CRRPGNKTVVPITLMSGLVFHSQPINKRPKQAWC 34	7
NARI-8	A2	R5	NSI	CKRPGNKTVVPITLMSGLVFHSQPINKRPRQAWC 34	7
NARI-9	B2	R5	NSI	CKRPGNKTVVPITLMSGLVFHSQPINKRPRQAWC 34	7
NARI-10	C1	R5	NSI	CKRPGNKTVVPITLMSGLVFHSQPINERPRQAWC 34	5
NARI-11	C2	R5	NSI	CKRPGNKTVVPITLMSGLIFHSQPINTRPRQAWC 34	6
NARI-12	B3	X4/R5	SI	CKRPGNKTVLPITLMSGLVFHSQPINTRPRQAWC 34	6
NARI-13	C3	X4/R5	SI	CKRPGNKTVVPITLMSGLVFHSQPINKRPRQAWC 34	7
NARI-14	C3	R5	NSI	CKRPGNKTVVPITLMSGLIFHSQPINTRPRQAWC 34	6
NARI-15	–	R5	NSI	CKRPGNKTVVAITLMSGLVFHSQPINARPRQAWC 34	6
NARI-16	–	R5	NSI	CKRPGNKTVVPITLMSGLVFHSQPINKRPRQAWC 34	7
NARI-17	–	R5	NSI	CKRPGNKTVVPITLMSGLVFHSQPINKRPRQAWC 34	7
NARI-18	–	R5	NSI	CKRPGNKTVVPMTLMSGLIFHSQPINNRPRQAWC 34	6

Disease staging of HIV-2-infected individuals and coreceptor usage, syncytium induction, amino acid sequences and net charge of the V3 *env* region of 18 Indian HIV-2 isolates.

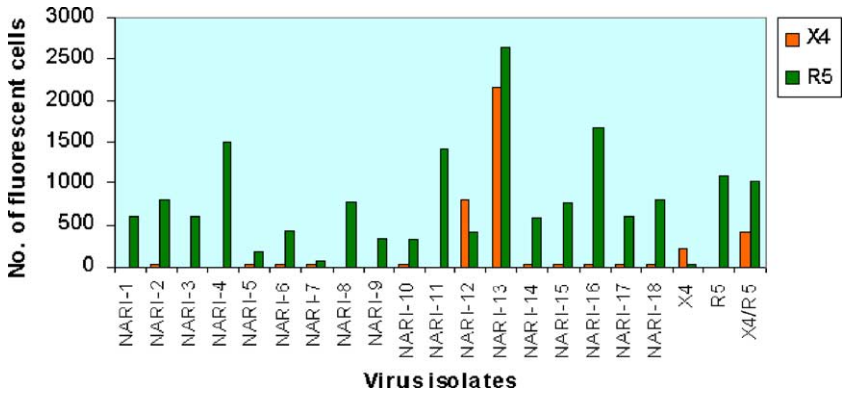

Fig. 1. Infection of GHOST-CXCR4/CCR5 cells with Indian HIV-2 isolates (NARI-1 to NARI-18) and standard positive controls (92/UG/029—CXCR4, 93/UG/082—CCR5 and 93/BR/020—CXCR4/CCR5) from NIH AIDS Research and Reference Reagent Programme.

four isolates in the late stage used CXCR4/CCR5 coreceptors and two used CCR5 coreceptor. In addition, one individual who presented with pulmonary tuberculosis but had normal CD4 count (830 cells/mm^3) used CCR5 coreceptor. Isolates from four individuals for whom CD4 counts were not available used CCR5 coreceptor (Fig. 1, Table 1).

Syncytium induction by HIV-2 isolates

The ability of HIV-2 isolates to induce syncytia was assessed using MT2 cell line for assigning SI or NSI phenotype. Nine isolates from patients in the early stage were NSI. Two of the four isolates from patients in the late stage of HIV disease were dual tropic (X4/R5) and induced syncytia formation (as shown by presence of multinucleated cells with increase in cell size) and two were NSI. In addition, the virus from one individual who presented with pulmonary tuberculosis but had normal CD4 count (830 cells/mm^3) was NSI. The isolates from four individuals for whom CD4 counts were not available were NSI (Table 1).

The ability of HIV-2 isolates to induce syncytia in other cell lines like SupT1 and U-937 was also assessed. The syncytium induction was more in SupT1 cell line compared to MT-2 cells. Eight isolates induced syncytia in SupT1 cell line while the remaining 10 were found to be NSI. There was no correlation between the disease stage and syncytium induction in SupT1 cell line. The isolates did not induce syncytia in U-937 cell line (data not shown).

Subtype identification

The nucleotide sequences were analyzed on HIV Basic Blast (www.hiv-web.lanl.gov) provided by Los Alamos HIV database. The blast analysis of all 18 sequences showed maximum homology with subtype A. The phylogenetic tree constructed using V3 sequences from representative subtype A (U05359), subtype B (AF170058) and subtype G (AF208027) further confirmed our isolates as subtype A (Fig. 2). The tree also revealed maximum homology of our sequences with sequences from western India (U07104) and southern India (AY081795) as compared to the sequences from eastern India (AY309063).

V3 sequence alignment

The consensus V3 region sequence for each of the isolates was determined and aligned to HIV-2$_{ROD}$ reference strain (M15390). The consensus sequences were compared with other published Indian sequences from western, southern and eastern India (Fig. 2).

Comparison of our HIV-2 isolates with HIV-2$_{ROD}$ revealed mean percent homology of 83% (range, 80–86%). The mean percent homology with isolates from western India was 96% (range, 94–100%), with isolates from southern Indian sequences was 95% (range, 93–99%) and with an isolate from eastern India was 87% (range, 85–91%). The genetic distance was calculated by Kimura 2 parameter method (http://www.ebi.ac.uk/clustalw/). The mean genetic distance of our HIV-2 isolates as compared with isolates from western and southern India was 3% (range, 0–6%) and with an isolate from eastern India was 13% (range, 9–16%).

In the V3 region sequences of Indian HIV-2 isolates, it was observed that there was deletion of two amino acids (H and Y) located at position 23 and 24 of HIV-2$_{ROD}$ sequence. The amino acid (AA) residue K of HIV-2$_{ROD}$ at positions 2 and 7 was replaced by R in two and three isolates, respectively. The AA residue K (at position 10) was replaced by V in 17 isolates and by L in one isolate. In 17 isolates, the AA residue Q at position 11 was replaced by P and by A in one isolate. Comparatively AA residues T and L were found to be conserved in all 18 isolates, even though they differed when compared with HIV-2$_{ROD}$ (M and H at positions 13 and 18). The AA residue at positions 19 (V replaced by I), 32 (R replaced by K) and 34 (A replaced by C) also differed in four, five and in one isolate, respectively, when compared with HIV-2$_{ROD}$. The AA residue K (at position 29) was replaced by N in three isolates, by each T and K in six isolates and by A, D and E in one isolate each.

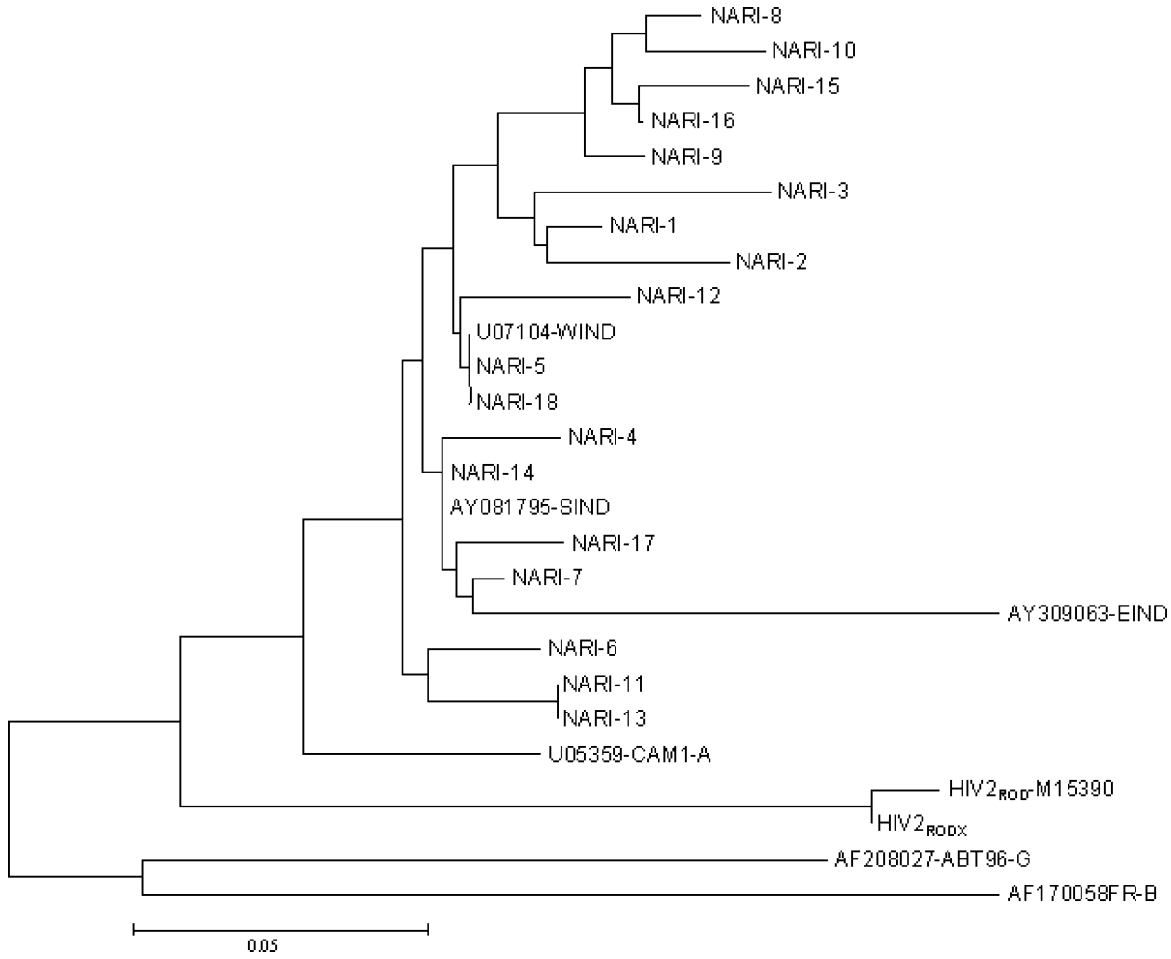

Fig. 2. Phylogenetic relationship of Indian HIV-2 isolates with representative isolates from subtype A (U05359), subtype B (AF170058), subtype G (AF208027), HIV-2_{ROD} (M15390), HIV-2_{RODX} (X05291) and published Indian isolates from west (U07104), south (AY081795) and east (AY309063) India. The tree was constructed by neighbour joining method using MEGA 2.1 software.

Additionally, the amino acids (A and W) at positions 32 and 33 were deleted in one isolate (Table 1, NARI-5).

Our study also demonstrated that CXCR4/CCR5 usage and syncytium induction were not associated with substitution of positively charged amino acids at positions 9 and/or 10 in the V3 region (Table 1). No isolate had positively charged amino acid residues at these positions. The mean positive net charge was 6.2 (range, 5–7). There was no difference between the mean positive net charge of dual tropic and SI isolates (mean = 6.5, range, 5–7) and CCR5 tropic and NSI isolates (mean = 6.2, range, 6–7). The potential N-linked glycosylation site (amino acid positions 6–8) within V3 region was conserved in all our isolates.

Discussion

HIV-2 epidemic is a public health problem in countries like Portugal (Soriano et al., 2000., UNAIDS/WHO, 1998) and Spain (Machuca et al., 1999), constituting 10–13% and 5% of all HIV infections, respectively. High prevalence of HIV-2 (around 10% of all HIV infections) has been reported in two rural communities of Senegal and New Guinea Bissau of West Africa (Lagarde et al., 2003; Norrgren et al., 1999) as well as in Angola and Mozambique (Schim van der Loeff and Aaby, 1999). The highest prevalence of 28% has also been reported among commercial sex workers in the Gambia (Ghys et al., 1997; Langley et al., 1996). Although, HIV-2 prevalence is low in other parts of the world, new cases continue to be reported. Since the first report in the year 1991 (Rubsamen-Waigman et al., 1991), HIV-2 infection has been reported from different regions of India (Arora et al., 2004; Kannangai et al., 1999; Mukhopadhyay et al., 2001; Rubsamen-Waigman et al., 1994).

As compared to HIV-1, HIV-2 infection is associated with long clinical latency, slow disease progression, low viral load and reduced transmission rate (Kanki et al., 1994; Marlink et al., 1994). The HIV-1 pathogenicity has been found to be associated with the genotypic and phenotypic characteristics of virus, which could be related to variations in the V3 region of *gp* 120 envelope protein (Hoffman and Doms, 1999; McDonald et al., 2001). Some of these variations may result in alteration of virological characteristics such as replication kinetics, syncytium inducing capacity and cell tropism.

Emergence of CXCR4 HIV-1 phenotypes is usually associated with CD4 cell decline and faster disease progression (Philpott, 2003). Our study revealed that two of the four isolates from individuals in late stage disease were dual tropic and efficiently induced syncytia in MT2 cells. The isolates obtained from individuals in the asymptomatic HIV disease stage were CCR5 tropic and NSI. When individuals were classified using WHO clinical staging system, 10 isolates obtained from HIV-2-infected individuals in clinical stage 1 and clinical stage 2, utilized CCR5 coreceptor. In case of the remaining eight isolates obtained from individuals in clinical stage 3, two isolates utilized both CXCR4 and CCR5 coreceptors. These results are in agreement with the earlier study by Morner et al. (1999) where seven isolates were obtained from AIDS patients. One of these seven isolates utilized X4 coreceptor, one used both X4/R5 and five utilized R5 coreceptor. However, Sol et al. (1997) have studied coreceptor tropism of seven isolates, which included two X4 isolates from AIDS patients and five R5 isolates from asymptomatic individuals, indicating that isolates from AIDS patients use X4 coreceptor only.

In this study, 16 of 18 HIV-2 isolates were R5 tropic. The predominant use of CCR5 by HIV-2 is in agreement with previous reports (Heredia et al., 1998; Morner et al., 1999) but differs from other studies in which all HIV-2 isolates were found to be CXCR4 tropic (McKnight et al., 1998). The frequent use of CCR5 by Indian HIV-2 isolates could be explained by several possible causes. Higher expression of CCR5 in the Indian population, as reported earlier by Ramalingam et al. (2002), may provide replication advantage to R5 HIV strains over X4 HIV strains, explaining the lack of shift in the viral phenotype during disease progression. Up-regulation of CCR5 expression as a result of larger loads of concomitant infections in developing countries like India may result in continued selection of R5 strains. *Mycobacterium tuberculosis* infection, which is the most common opportunistic infection in India with 50–60% HIV-infected persons developing tuberculosis, has been reported to cause up-regulation of CCR5 (Fraziano et al., 1999; Morris et al., 2001; Shalekoff et al., 2001). The frequent use of CCR5 could also be due to down-regulation of CXCR4 expression due to circulation of activated T lymphocytes thereby affecting propagation of CXCR4 strains (Bermejo et al., 1998).

The HIV-2, subtype A accounts for a majority of HIV-2 infections and is the predominant subtype in New Guinea Bissau and Europe (Norrgren et al., 1997; Schim van der Loeff and Aaby, 1999). Occurrence of subtype B has been reported from eastern parts of West Africa (Ghana and Ivory Coast) and occasionally from Europe (Schim van der Loeff and Aaby, 1999). Although prevalence of HIV-2 (around 0.02%) is low in Sierra Leone, occurrence of subtype A, subtype B, subtype E and subtype F has been reported from this region revealing highest genetic diversity (Reeves and Doms, 2002). Based on the sequence analysis of the V3 region, our studies revealed that globally distributed HIV-2 subtype A is the predominant strain in western part of India.

The occurrence of HIV-2 subtype A has been reported earlier from western India (Grez et al., 1994), southern India (Kannangai et al., 2003) and eastern India (Bhanja et al., 2004) in samples collected during the years 1991–1992, 1998–2001 and 2003, respectively. Comparison of the percent homology (96% and 95%), mean genetic distance (3%) and phylogenetic analysis revealed that our sequences are closely related to the sequences of the isolates from western and southern India, respectively. Less diversity in the circulating HIV-2 from western India could be attributed to low transmission rate of the virus and also due to introduction of single genotype (subtype A).

Analysis of the V3 sequences revealed that there was no association between the net charges and the syncytium induction or coreceptor usage. It was also observed that the amino acid residues at positions 9 and/or 10 (313 and/or 314 in the *gp*105 region) did not exhibit positive charge. The consensus sequences were obtained from three clones from each isolate. The amino acid residue F at position 20 was replaced by S in two clones from an isolate. The amino acid residues QAW, at positions 31–33, were replaced by PGM in one clone obtained from one of the two X4/R5 isolates (NARI-12). The amino acid residue H at position 21 replaced by D in one clone from the second dual tropic isolate (NARI-13). However, no replaced residue was positively charged and hence did not increase the overall positive charge of the V3 loop. Detailed analyses of the sequences obtained from three clones from both dual tropic isolates revealed that none of them had increased positive charge and truly represented the sequences of dual tropic viruses. This is not in agreement with the findings of Albert et al. (1996) and Morner et al. (1999) who have reported correlation between the net charge and the presence of positively charged amino acid at positions 9 and 10 in the V3 region of HIV-2 and CXCR4 usage. Thus, charge on the amino acid residues at positions 9 and/or 10 may not be predictive of the SI phenotype or of the CXCR4 coreceptor usage of the Indian HIV-2 isolates.

In summary, the present study indicated that R5 phenotype is the predominant phenotype among Indian HIV-2 isolates recovered from patients in the early stage. Two of the four HIV-2 isolates obtained from late stage patients exhibited shift from R5 to X4/R5. Our study revealed predominance of globally distributed HIV-2 subtype A strain in western part of India. The study also demonstrated lack of association between the net charge, positively charged amino acids in the V3 region of *gp*105 glycoprotein and the syncytium induction or coreceptor usage.

Materials and methods

Study subjects

After obtaining informed consent, relevant demographic and clinical information was collected and blood sample was drawn from 18 HIV-2-positive individuals residing in

western region of India during the years 1995 to 2001. The individuals were found positive for HIV-2 elsewhere and were then referred to our clinic. The HIV-2-positive status of these individuals was confirmed using ELISA as an initial screening test (Labsystems HIV EIA, Finland and Detect EIA, Canada). The positive samples were confirmed by a rapid test (HIV Tridot, India) and Western blot (Cambridge Biotech, Ireland and Innolia HIV Confirmation, Belgium).

Determination of HIV disease status

The patients were classified for HIV disease stage as per CDC AIDS Surveillance case definition, 1993 (CDC, 1992) considering absolute CD4 counts and clinical presentation. Using these criteria, 13 patients could be classified for whom CD4 counts were available. In addition, one individual who presented with pulmonary tuberculosis but had normal CD4 count (830 cells/mm^3) could not be classified as having late stage HIV disease. The remaining four patients were assessed for HIV related symptoms. The CDC classification system was utilized for data analysis.

The patients were also classified for disease stage using WHO clinical staging system for HIV/AIDS (WHO, 2003), since CD4 counts were not available for four individuals.

Determination of CD4 counts

The absolute CD4 cell counts were estimated on freshly collected blood samples by two-color analysis using anti-CD4 antibodies conjugated with phycoerythrin (Becton Dickinson, San Jose, CA, USA). The results were analyzed on a FACSort flow cytometer (Becton Dickinson, San Jose, CA, USA). The absolute CD4 counts were calculated utilizing the total leukocyte counts obtained by the manual method using Neubaur's chamber.

Virus isolation and virus stock development

The viruses from HIV-2-infected individuals were isolated as described elsewhere (Kulkarni et al., 1999). Culture supernates collected on 14th and 28th post infection (PI) day were centrifuged and filtered through 0.22 μ syringe filter and stored at −70 °C. The culture supernates were used for passaging the virus further (Passage 1) by infecting activated heterologous normal lymphocytes for confirmation of HIV-2 in culture using polymerase chain reaction (PCR). The cultures were incubated for 14 days at 37 °C in 5% CO_2 and humidified incubator. The cells and supernates were collected on 7th and 14th PI day.

The Standard strains (92/UG/029, 93/UG/082, 93/BR/020 and CMU02) were obtained from NIH AIDS Research and Reference Reagent Programme. Stocks of these isolates were prepared in PHA-P activated PBMCs from HIV negative, heterologous donor and used as controls for coreceptor determination and syncytium induction assays.

Polymerase chain reaction

DNA was extracted from infected PBMCs collected on 7th and 14th PI day (Passage 1) using ISOQUICK Nucleic acid Extraction kit (ORCA Research Laboratory Ltd., USA) as per manufacturer's instructions and stored at −20 °C until further use. Amplification of 1.5 kb fragment of *env* gene of HIV-2 was performed using nested PCR as described elsewhere using primers derived from HIV-2_{ROD} (Kulkarni et al., 1999).

Cell lines

The GHOST cells (human osteosarcoma cells expressing CD4 and CXCR4/CCR5 coreceptor along with green fluorescent protein (GFP) as the reporter gene driven by HIV-2 *tat* as a promoter) were obtained from Dr. Zolla-Pazner, New York Veterans Affairs Medical Centre, NY, USA. The cells were maintained in Dulbecco's Modified Eagles Medium (DMEM, Sigma, USA), containing 10% FCS and 100 U/ml Penicillin, 100 μg/ml Streptomycin, 500 μg/ml Geneticin, 25 μg/ml Hygromycin and 1 μg/ml Puromycin (Sigma, USA).

The T lymphoid cell lines—MT2 and SupT1, and U-937 (a macrophage cell line) used for determining syncytium induction were obtained from NIH AIDS Research and Reference Reagent Programme. The cell lines were maintained in RPMI containing 10% FCS and 100 U/ml Penicillin, 100 μg/ml Streptomycin, 80 μl/ml Gentamycin. The cell lines were incubated at 37 °C in 5% CO_2 and humidified incubator.

Coreceptor usage using GHOST cells

GHOST cells expressing CXCR4 or CCR5 coreceptors were used for determining coreceptor usage. The assay was carried out as described by Cecilia et al. (2000). Briefly, GHOST cells expressing CXCR4/CCR5 seeded at a concentration of $6x10^4$ cells/well/0.5 ml medium in the 24-well tissue culture plates, were allowed to grow for 24 h, when at least 70% confluency was seen. The cells were infected in duplicate with 1:2 diluted virus stocks in presence of 8 μg/ml DEAE-Dextran (Sigma, USA). After overnight infection, the cells were washed, fed with the growth medium and incubated for 3–4 days at 37 °C in 5% CO_2 and humidified incubator. The harvested cells were re-suspended in 1 mM EDTA, fixed in 2% formaldehyde and analyzed by FACSort flow cytometer (Becton Dickinson, San Jose, CA, USA). The fixed cells were gated based on forward-scatter and side-scatter. The number of infected cells was determined using scattergram of fluorescence on the *x* axis versus forward-scatter on the *y* axis after setting the gate with uninfected cells. A total of 15,000 events were scored. The mean number of fluorescent cells in the uninfected cell cultures+3 SD was considered as the cut-off value, which was 12+20 cells for GHOST-CXCR4 cells and 14+28 cells

for GHOST-CCR5 cells. The cut-off value was determined based on 20 observations in a total of five assays. Isolates demonstrating number of fluorescent cells more than the cut off value were considered as positive for virus infectivity and coreceptor usage. Results were compared with the standard strains 92/UG/029 (CXCR4), 93/UG/082 (CCR5) and 93/BR/020 (CXCR4/CCR5) obtained from NIH AIDS Research and Reference Reagent Programme.

Syncytium induction in T lymphoid cell lines

The MT2, SupT1 and U-937 cells were washed and counted. 2×10^4 cells were seeded in each well of the flat bottom, 96 well plates (Costar, USA). 50 μl of positive culture supernate (passage 1) was added to the respective wells in triplicate. The volume was adjusted to 200 μl/well and the plates were incubated for 14 days at 37 °C in 5% CO_2 and humidified incubator. The cells were fed twice a week with fresh medium and split once a week. The syncytium induction was recorded microscopically on every 4th PI day. Standard strain CMU02, which induces syncytium in MT2 and SupT1 cell lines, was included as a positive control. The cytopathic effect (CPE) produced by Indian isolates was compared with CPE produced by CMU02 and then graded accordingly.

Cloning and sequencing

The amplified *env* gene was cloned into $PCR^{2.1}$ (Invitrogen, USA) and $P^{TZ57R/T}$ (MBI Fermentas, Russia). The clones were confirmed by restriction digestion following kit manufacturer's instructions and three clones were obtained for each HIV-2 isolate. A 102-bp V3 region of HIV-2 envelope gene was sequenced using H2-14 primer—5′ AGC CAT GCA GGA GGT GAA 3′. The fragments generated were aligned using clustal W/1.8 software. The alignment was utilized for construction of phylogenetic tree using V3 sequences from representative subtype A (U05359), subtype B (AF170058) and subtype G (AF 208027) and the MEGA 2 software (Kumar et al., 2001). The V3 sequences for subtype C, subtype D, subtype E and subtype F could not be obtained as these subtypes are confirmed based on the sequences from either *gag*, *pol* or *gp*41 regions of HIV-2 genome.

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