

Coinfection and Genetic Recombination between HIV-1 Strains: Possible Biological Implications in Australia and South East Asia

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Abstract

It has been recognised that human immunodeficiency virus (HIV) mutates rapidly and that nucleotide substitutions, deletions, insertions, and rearrangements resulting from recombination events are the main factors that result in variation of the HIV-1 genome. Together, these processes are actively contributing to the diversity and virulence of viral forms comprising the acquired immune deficiency syndrome (AIDS) pandemic. There are 9 HIV-1 subtypes recognised (A-H and O), based on the envelope region segments. Inter-subtype recombination has been already described, whereas intra-subtype recombination has been difficult to detect. In this study, we have identified in vivo genetic recombination between HIV-1 strains belonging to subtype B in a patient who presented both intravenous drug use (IVDU) and homosexual sex as risk factors. Genetic analysis of viral strains in the hypervariable V3 region of the envelope gene indicated the presence of three distinct sequence groups categorized according to their respective tetrapeptide motifs – GPGR, GLGR and GPGK. Detailed genetic and phylogenetic analyses suggested the recombination occurring only between sequence groups with GPGR and GPGK tetrapeptide motifs. These data suggest that coinfection with closely related strains can occur in vivo, and the generation of hybrid HIV-1 genomes via genetic recombination between subtype B strains can result in further antigenic diversity which may thwart diagnosis and future vaccine efforts. Since HIV-1 subtype B is still the most commonly found subtype around the globe, the hybrid genomes between different subtype B strains may result in epidemiologic shifts and altered pathogenesis.

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Introduction

One of the most remarkable features of retrovirus genetics is the high rate of genetic recombination.¹ Nucleotide substitutions, deletions, insertions, and rearrangements resulting from recombination between closely related or divergent strains of human and animal retroviruses are some of the most important means for generating genetic diversity. Such diversity may be a random process, or may allow the virus to counteract the host's immune pressure.

Genetic recombination between retroviruses can only take place within the same infected cell, if the cell is superinfected with multiple strains or subtypes. Although retroviruses (such as HIV-1) have been consid-

ered to be highly recombinogenic,² the frequency of recombinational events occurring in nature has been rarely documented. This is partially due to the reason that there is a paucity of evidence regarding the coinfections or sequential hyperinfection with different HIV-1 subtypes and/or strains in an individual given that the host's immune response may play a role in protecting against infection by a second HIV-1 strain. Thus, the likelihood of coinfection with multiple HIV strains in a patient is only during the seroconversion phase. As a consequence, the recombination between different HIV-1 subtypes or variants is likely to be a source of genetic variation in HIV-1, but the power of such natural events is undermined due to existence of fewer reports in the literature.

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Together, the intravenous drug users and people with multiple sexual contacts (especially promiscuous homosexuals) serve as a potential source of providing information regarding coinfection with multiple HIV strains/variants, and also the clues to possible genetic recombinational events *in vivo* either between different HIV-1 subtypes or within the same subtype. Present nomenclature suggests that there are nine distinct subtypes recognized for HIV-1 (A-H and O)² based on the envelope V3 region of HIV-1, and each of these subtypes are found either singly or multiply in various geographical regions of the world.^{2,3} Robertson et al,⁴ based on the previously published sequences² have shown that there are recombinant HIVs existing in nature which have contributed enormously to genetic diversity in HIV and simian immunodeficiency virus (SIV).⁵ Furthermore, there is also evidence describing the possibility of recombinant HIV-1 between subtype A/D in Sweden and subtype B and F in Brazil.⁷ Recent evidence also suggests that recombination occurs most commonly among divergent subtypes, and the likelihood of recombination has been speculated in the geographical regions where HIV-1 subtypes co-circulate.* More recently, Gao et al⁹ have described that a virulent subtype E, found predominantly in Africa and South East Asia (Thailand and India), is actually a recombinant virus with the envelope of subtype E recombined with the gag gene from subtype A. In Thailand where AIDS cases have risen dramatically in the past five years, HIV-1 subtype E has been found to be infecting proportionally more people. Whether this is due to increased pathogenicity from recombination or other molecular reasons is a point of debate.

So far, the reports mentioned above have described genetic recombination between divergent HIV-1 strains (intersubtype). In contrast, it has been difficult to demonstrate recombinational events between the same strains (intrasubtype) because they are not easy to detect.

In this study, we report a case of a promiscuous homosexual injecting drug user (IDU) (patient 890) who was coinfecting with multiple HIV-1 strains. We have analysed the V3 region clones derived from the patient's peripheral blood mononuclear cells (PBMCs), and the reasons for choosing the V3 region for analysing recombinants are several fold. First, there is substantial diversity in this region to discriminate between different variants. Second, subtyping of HIV-1 strains, based on the V3 region has been exhaustively defined. Third, the recombination between HIV-1 subtypes have been described based on the V domains. Fourth, the V3 loop of the envelope has received the greatest attention because it contains important epitopes involved in virological characteristics including fusion and virulence and cell tropism, as well as in potent immunological responses including neutralizing antibody and

cytotoxic T-cell responses.^{10,11}

Materials and Methods

Virus Culture

In 1990, patient 890 was identified with HIV-1 infection and a virus co-culture study was carried out in 1993 only. Due to the worsening condition of the patient he refused permission for future samples to be taken. 5×10^6 fresh PBMCs (available from September 1993) from the patient were co-cultured with an equal number of uninfected donor PBMCs that had been stimulated with phytohemagglutinin (PHA) and IL-2 for 48 hours using standard methods. Cultures were passaged on 10/9/1993; 19/9/1993; 20/9/1993, and 27/9/1993, respectively. The co-cultures were maintained twice weekly by addition of fresh PBMCs for a period of 8 to 11 weeks, and sampled at 3-day intervals for viral replication by the measurement of p24 antigen and particle associated reverse transcriptase (RT) activity. Integrated proviral DNA copies were assayed by polymerase chain reaction (PCR).

MT-2 Assay

Syncytium formation was determined on the MT-2 cell line. 1×10^6 infected PBMCs were co-cultured with 2×10^6 MT-2 cells in 4 ml of Iscove's modified Dulbecco's medium supplemented with 10% fetal calf serum, 0.03% glutamine, and antibiotics in a flask. The flasks were kept at 37°C in a 5% CO₂ incubator for 4 weeks and the cultures were checked twice a week under a microscope for the presence of multinucleated giant cells.

Proviral DNA Extraction and Polymerase Chain Reaction

The DNA was extracted from fresh PBMCs of the patient by using Quiagen (Quiagen, Germany) blood kit as per the manufacturer's protocol. One microgram (μ g) of genomic DNA from the infected cells was used in nested PCR by targeting primers in the V3 region of HIV-1. The V3 primers are listed in Table I. Twenty picomoles (pm) of each external and internal primer was used in PCR using 30 cycles with external set of primers, and 25 cycles with internal set of primers. Reaction conditions used have been described elsewhere.¹² Thermal cycling conditions were at 94°C for 5 minutes; 55°C for 1 minute and 72°C for 2 minutes (cycle 1) and at 94°C for 1 minute; 55°C for 30 seconds and 72°C for 2 minutes (30 and 25 cycles, respectively). Polymerase chain reaction amplified fragments were run on 1% agarose gel and visualised under ultraviolet light. The gel was denatured in 0.4M NaOH for 20 minutes and transferred onto a Nylon membrane (Du Pont, USA) overnight, followed by hybridization with a [γ -³²P]ATP labeled V3 detector probe, at 42°C overnight. The membranes were washed in $2 \times$ SSC and 0.1% SDS and exposed to X-ray Hyperfilm (Amersham, Australia)

and the films were scanned on an image scanner.

Cloning and Sequencing

5 µl of the PCR fragments was ligated into PGEM-T vector in 10 µl final volume (Promega, USA) and incubated at 15°C overnight. 4 µl of the ligation mix was used in transforming *Escherichia coli* (DHα F' strain). The transformed bacteria was plated onto Luria-agar plates with 75 µg/ampicillin/plate, 40 µg of X-gal, and 20 µg of IPTG, respectively. The plates were incubated at 37°C overnight, followed by a transfer of bacterial colonies onto Nylon membrane, denaturation and subsequent hybridization with the V3 specific detector probe. Positive clones were streaked onto fresh plates and individual clones were cultured overnight in Luria broth (10 ml) with ampicillin at 37°C under vigorous shaking (200 rpm). Recombinant plasmid DNA was extracted from the positive clones, purified and sequenced by di-deoxy chain termination method.

Loop Charge Calculations

The V3 loop charge for each clone was calculated using a DNA id+1.8 software (Fred Dardel, France) which assigns +1 charge to basic amino acid residues, -1 charge to acidic and zero to the remaining residues.

Computer Analyses

The V3 gene sequences were compared with previously known sequences and aligned by using PILEUP and phylogenetic analyses (based on peptide V3 sequences) were done using CLUSTAL¹³ and Neighbor joining method.¹⁴ The phylogenetic trees were bootstrapped with one thousand replications, and the branching nodes with 92% to 100% confidence values were considered reliable for comparisons.

Results

Cell Culture

The cultures, obtained from the patient tested positive for HIV-1 infection as tested by their reverse transcriptase and p24 antigen titres. No cytopathic effect (cpe) was observed *in vitro* in these cultures. Thus, predominantly most cultures were negative for any cytopathic effect.

Syncytium Formation

No syncytium formation was detected in three out of four cultures using the MT-2 assay. The virus recovered from culture done on 20/9/1993 showed syncytium formation. The viruses recovered from cultures done on 10/9/1993; 19/9/1993 and 27/9/1993 revealed the presence of non-syncytium inducing (NSI) strains.

Sequencing and Phylogeny

We could successfully amplify 320 base pair (bp) fragment encompassing the entire V3 loop region

(Fig. 1). Comparison of 11 different clones, over 320bp, with the prototypic HIV-1 (pNL43) indicated a minimum pairwise difference of 11% and maximum pairwise difference of 15.3% (data not shown). In contrast, the average pairwise difference between clones was 5.74% with a minimum pairwise difference of 1% (data not shown).

As inferred from nucleotide sequence, the peptide sequence comparisons also showed close relationship between clones with unique sequences. Only one clone v32-6 showed a premature stop codon in the V3 region at aa residue 64, along with unique changes at residues 51, 43 and 44 (Fig. 2).

Interestingly, V3 loop region sequences were segregated into 3 different tetrapeptide motifs [GPGK (5 clones), GPGR (5 clones) and GLGR (1 clone)], characterized by 4 different octapeptide motifs [PIGPGKAF, HIGPGKAF, PIGLGRAF and PIGPGRAF] (Fig. 3). Although all V3 clones (Fig. 2) showed a number of changes in the flanking regions of the octapeptide motif (between aa 32-35 and aa 44-45), distinct changes were noted in clones v31-3, v32-8 and v32-5 at these positions. In comparison to the prototype (HIV-1 pNL43), another interesting change which occurred consistently in all 11 clones was identified between aa residues 59-63 where residues ISRAQ (found in the prototype) changed to LNGTE (Fig. 2). Interestingly, this L (I) NGTE(K) change is found consistently in consensus subtype E sequence, and in only two strains of subtype B (BR3 RJ623, and CM CA5) from Brazil (LNGTK) and Cameroon (LNGTQ), respectively.² Although the significance of these changes in HIV-1 pathogenesis is not clear, the genotypic data presented here suggest the presence of multiple HIV-1 strains in the patient's PBMCs, some of which may have either evolved *via in vivo* recombinational events or were acquired prior to initial infection with HIV.

Recently, arginine [R] at position 11 (Fig. 2) has been implicated in cpe¹² and determining SI phenotype.¹⁰ We have also observed the presence of R at this position in clones v31-3 and v32-8, whereas in the remaining 9 clones this R change was absent. In fact, the presence of

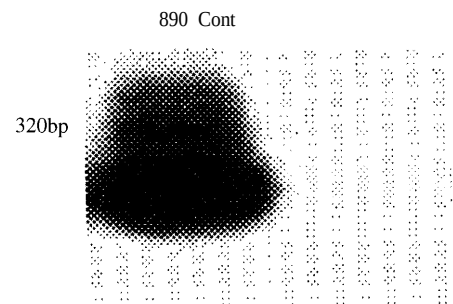


Fig. 1. Southern blot hybridization of the polymerase chain reaction amplified V3 region 320 base pair fragments. Cont: primer or no DNA control.

	V3 Loop										V3 Loop Charge and Hydrophilicity	
	1										35	
pNL43v3	SDNFTNNAKTIIVQLNESVAIN	CTRPNNNTRKSIYIGPGR	AFHTTGRIIGDIRKAHC								6	0.1570
890v31-1	D I V	D TP K	YH D Q Q								2	0.2280
890v31-3	D I V	YK QR P	Y Y G K Q Q								6	0.1140
890v31-5	D RI V	R T P L	YH D R Q Q								4	0.1820
890v32-1	D I V	TP	FH D Q Q								3	0.1420
890v32-2	D I V	TP	FH D Q Q								3	0.1420
890v32-3	D I V	TP K	YH D Q Q								3	0.1480
890v32-4	D I V	TP K	YH D Q Q								3	0.1480
890v32-5	D I V	TH K	YH D Q Q								3	0.1340
890v32-6	D I V	TP	FH D Q Q								4	0.0200
890v32-8	D I V	YK QR P	YY G Q Q								5	-0.0220
890v32-9	D I V	TP K	YH D Q Q								3	0.1480

pNL43v3	N	ISRAQ	WNNTLEQIVKKLREQFGNNKTIVFNQSSGG
890v31-1	LNGTE	K A	V V-
890v31-3	LNGTE	D E IT	K V- I
890v31-5	LNGTE	K A	V V-
890v32-1	LNGTE	K A	V H V-
890v32-2	LNGTE	K A	V V-
890v32-3	LNGTE	K A	V V-
890v32-4	LNGTE	K A	V V-
890v32-5	LNGTE	K A	V V-
890v32-6	LNGTE *		
890v32-8	LNGTE	K A	V V-
890v32-9	LNGTE	K A	V V-
Con.st-B	LSRAK		
Con.st-C	ISKDK		
Con.st-D	ISGAE		
Con.st-E	INGTK/E		
Con.st-F	VSGTQ		
Con.st-GVSKTK			
Con.st-HITRED			
Con.st-OYNTTD			
Con.st-UISRTK			

Fig. 2. V3 region amino acid sequences of 11 different clones derived from patient 890. On/y amino acid differences are shown in each case. The changes and hydrophilicity values correspond to only V3 loop region (Cys1-Cys35) for each clone.

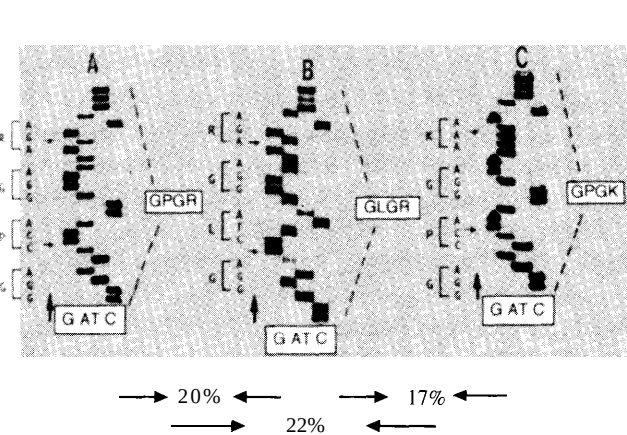


Fig. 3. DNA sequencing by dideoxy chain termination method depicting the presence of three distinct V3 loop tetrapeptide motifs (GPGR, GLGR and GPGK) found in the patient's PBMCs. GATC is the orientation of bases in ascending order. On the top of each group are their respective peptide sequences showing the percent differences between each group.

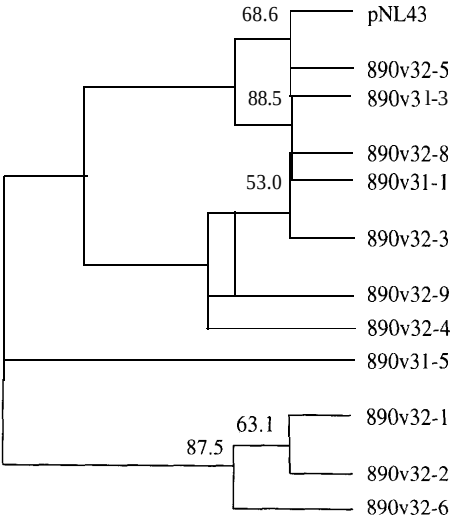


Fig. 4. Phylogenetic analysis of 11 clones derived from the patient's PBMCs. The tree was bootstrapped with 100 replications. The values shown at each branch point are bootstrap values.

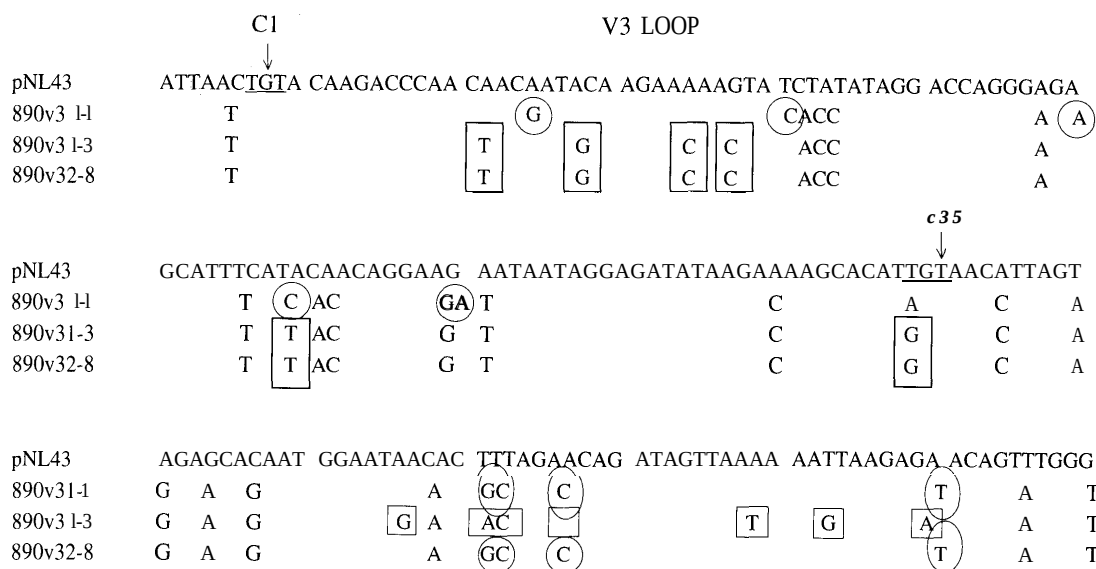


Fig. 5. Genetic recombination mechanism. Three-way nucleotide sequence alignment of clones 890v31-1, 890v31-3 and 890v32-8 showing the phenomenon of genetic recombination. The bars within the circles and rectangles are identical bases common between two clones. In the first 120 bases, clones 31-1 and 32-8 are identical, whereas in the last 60 bases, clones 31-1 and 32-8 become identical. C1 and C35 are cysteine residues which form the V3 loop.

R-11 conferred a high charge to the V3 loop. Moreover, the high' charge was confined only to clones (v31-3-charge 6 and v32-8 charge 5) where R-11 change was present (Fig. 2). Although the two clones [v31-3 and v32-8] are 99% identical in the V3 loop region, owing to substitution of I→K (at position 27) conferred charge 1 to clone v31-3. Overall, a predominance of low V3 loop, charge (between +2 and +4) was seen in the other 11 clones. Hydropathicity values also suggest that these fluctuations in V3 loop charge may have implications at the antigenic presentation level.

The phylogenetic analysis of peptide sequences by the Neighbour joining method¹³ (Fig. 4) further suggested a marked segregation between HIV-1 strains present in the patient's PBMCs. These relationships between different clones further supported infection with multiple HIV-1 strains.

Genetic Differences Between GPGR, GLGR and GPGK Groups

Comparison of the V3 amino acid sequences showed that the sequence group GPGR was divergent from GLGR by 20%, GLGR from GPGK by 17.1%, and GPGR from GPGK by 22.8%. Thus, the patient had three distinct groups in the V3 region (Figs. 2 & 3).

Mechanism of Recombination

Three-way alignment of three individual clones (890v31-1, v31-3 and v32-8) from patient 890 revealed that there was exchange of genomic regions within the V3 region. The first 120 bases in clones v31-3 and v32-8

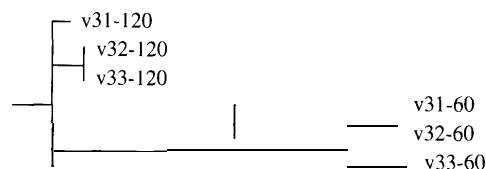


Fig. 6. Phylogenetic segmental analysis of 2 segments of the V3 region (120bp and 60bp) between which the break-point was identified (Fig. 5). This figure shows clearly that clones v31-3 and v32-8 are identical based on 120 base pairs and v31-1 diverged out, whereas based on 60 base pairs clones v31-1 and v32-8 become identical and clone v31-3 diverged out. The tree was bootstrapped with 1000 replications with each branch point having 100% bootstrap value.

were identical, whereas clone v31-1 was divergent. In the remaining 60 bases of the V3 region, clones v31-1 and v32-8 became identical, whereas clone 32-8 diverged out (Fig. 5). The recombining clones belonged to GPGR and GPGK tetrapeptide motif groups. Individual segmental phylogenetic analysis of 120 bases and 60 bases further suggested similar relationships seen at the nucleotide level, with 100% bootstrap values for each branch point (Fig. 6). Detailed phylogenetic analyses (Fig. 4), using the Neighbour joining method and bootstrapping, suggested 'strongly the relationship between the GPGR and GPGK groups. In contrast, the GLGR group diverged out as an outlier.

Discussion

In this study we describe a promiscuous homosexual IDU coinfecting with multiple closely related HIV-1 strains. Our *in vivo* genetic and phylogenetic analyses together suggested the recombination between HIV-1 strains belonging to the subtype B.

The molecular changes in the hypervariable V3 loop region play an important role in HIV-1 infection and cell tropism, which in turn can modulate pathogenesis of

HIV-1 *in vivo*. Also the importance of V3 tip motifs (both tetra and octapeptide) in viral fusion function has been previously described.¹³ The V3 sequences presented in this study were concordant with episodic HIV-1 infection with multiple HIV-1 strain, which are genetically related. Theoretically, it is unlikely that after initial exposure to HIV-1 infection a second infection with another strain is possible owing to a strong immune response mounted against the HIV-1 antigens. Since the patient was a promiscuous homosexual, it is likely that he was possibly infected with a swarm of viruses, or independently on two different occasions before seroconversion.

Furthermore, just before the death of the patient, coinfection was also evident from the presence of three distinct groups of V3 sequences showing three different V3 tetrapeptide motifs (tetrapeptide-GPGR, GLGR, GPGK; octapeptide-PIGPGRAF, PIGPGKAF, HIGPGKAF and PIGLGRAF), and also from the charge distribution. In addition, given the overall incidence of these tetra and octapeptide motifs in different HIV-1 subtypes it became apparent that although all three V3 tip motifs (especially GPGR) are commonly found in subtype B, they can rarely be found in other subtypes such as D, E, F, A, and U. Generally, the occurrence of GPGK and GLGR motifs in HIV-1 subtypes are rare. In contrast, the octapeptide motifs observed here have not been reported in the database except for HIGPGRAF.² In fact, the occurrence of P in octapeptide is extremely rare. In addition, the occurrence of amino acid stretch LNGTE (aa36-40) is found commonly in subtype E [I(L)NGTE(K)] but predominantly absent in subtype B except for HIV-1 BR3 and CA5 from Brazil and Cameroon, respectively. In our patient (patient 890), the fact that the patient harboured recombinant virus was well supported by the V3 loop peptide comparisons of GPGR, GLGR and GPGK sequence groups in the patient, and also by our three-way nucleotide alignment and phylogenetic analysis of peptide sequences, which further suggested distinct genetic relationships between the three individual clones derived from the patient's PBMCs.

Recently, it has been suggested that uncharged amino acid residues at position 11 and 25 of the V3 loop contribute to NSI phenotype, whereas a charge in either one of them gives rise to SI phenotype.^{10,11} Interestingly, R-11 has been suggested to be crucial in cpe¹⁵ and in syncytium induction.¹¹ In our study this is further supported by the presence of R-11 in 2/11 clones, which is concordant with our culture results where only 1 of 4 cultures revealed syncytium formation.

Previously, higher loop charge has been implicated in T-cell tropism and variants with higher loop charge have been known to emerge during HIV-1 pathogenesis.¹¹ Also, according to a previous report the appearance of SI variants is associated with a rapid decline in CD4+

cells and onset of AIDS.¹⁰ Further study of biological clones of naturally occurring virus isolates is needed to elucidate this fact. It may be that other genomic regions such as V1, V2, V4 and V5 are also important in HIV-1 pathogenesis. In sum, our data do not agree with the hypothesis that positively charged amino acid residues at positions 11 and 25 of the V3 loop contribute to SI phenotype.¹¹ The most likely biological explanation for the death of patient 890 may have been either due to infection with multiple HIV-1 NSI and SI strains, or owing to the presence of recombinant viruses *in vivo*. Our data clearly demonstrate the presence of recombinant virus *in vivo*. Further biological studies are needed to confirm if such recombinant strains are being transmitted. And if they are, then is there any evidence of altered pathogenesis in those patients which can be correlated with the presence of recombinant strains.

Conclusions

HIV-1 coinfections and possible recombination, *in vivo*, could pose a further challenge to the complex task of formulating a vaccine against HIV. It is conceivable that *in vivo* recombinational events between different HIV-1 strains can result in variants with previously or unrecognizable neutralization epitopes, especially in instances where the epitopes are discontinuous rather than linear. Future studies are necessary to assess the extent of this phenomenon. With the recent global trafficking of HIV variants in different parts of the globe (Fig. 7), it is likely that new patterns will emerge leading to creation of recombinant HIV-variants, with altered pathogenesis (Fig. 8).

The infection with recombinant HIV-1 strains in humans raises a number of important issues with regard to their pathogenicity and also the epidemiologic shifts in

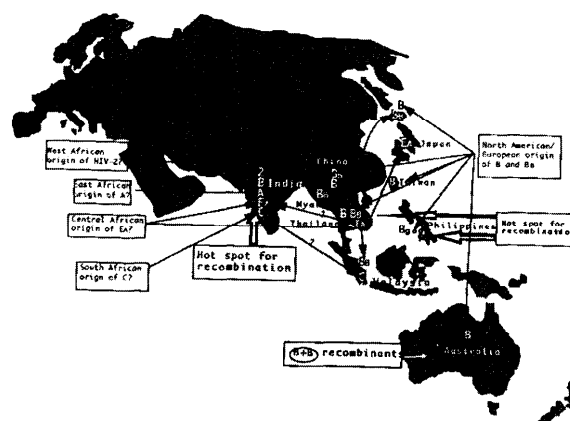


Fig. 7. HIV-1 epidemiologic patterns in South East Asia and India showing the co-circulation of multiple subtypes in various geographical regions, their dissemination to other parts. Hot spots for recombination are regions where either the recombinant HIV-1 strains have been reported (Thailand: recombinant between subtype E and A) or recently been detected.

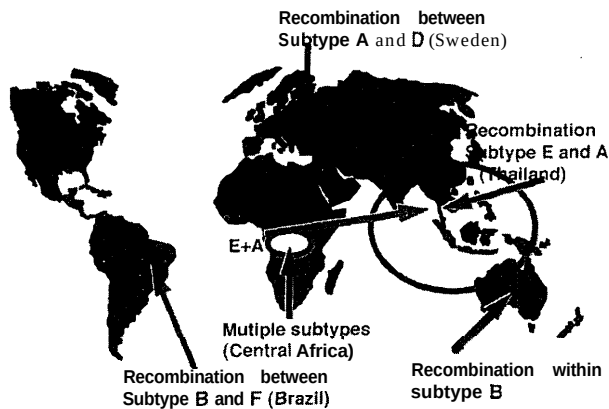


Fig. 8. Global epidemiology and distribution of recombinant HIV-1 strains.

Australia and South East Asia. The biologic effects on the host through the interaction of two different or related subtypes may be additive, synergistic, or inhibitory. The impact of recombination on the clinical course of HIV-1 disease can only be ascertained by the prospective study of a population of incident cases and biological activity of the recombinant and non-recombinant viruses.

The infection in Thailand with recombinant subtype E+A recombinant HIV-1 has resulted in large number of AIDS-related deaths. Apart from subtype B, subtype E+A recombinant is the only HIV-1 strain that has established itself in a particular population. Molecular reasons for its pathogenicity are unclear, but it seems that subtype E viruses are spreading with extraordinary pace. Future epidemiologic shifts are expected in the neighbouring countries of South East Asia. For instance, if subtype E+A recombinant establishes itself in India where subtype B, C, A and also HIV-2 are resulting in AIDS-related deaths, more aggressive patterns of HIV-1 epidemiology may emerge. Since subtype E viruses are predominantly transmitted via heterosexual contact,^{16,17} it will be devastating for South East Asia where HIV-1 transmission is largely heterosexual.

The altered pathogenesis and changing epidemiologic patterns of HIV-1 infection resulting *via in vivo* recombinational events between the same and/or different HIV-1 subtypes may have important implications for future HIV vaccine development.

Since HIV-1 infections are on the rise in South East Asia, the data on transmissibility and extent of genetic recombination happening between HIV-1 variants in Australia and South East Asia seem warranted. Con-

tinuous surveillance of HIV-1 variation worldwide will be imperative.

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