T-cell line adaptation of human immunodeficiency virus type 1 strain SF162: effects on envelope, vpu and macrophage-tropism

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Changes in co-receptor-use by human immunodeficiency virus type 1 (HIV-1) strains are relatively rare in vivo. Here we describe two variants derived from the CCR5-using strain SF162, selected for replication in the C8166 T-cell line. Amino acid substitutions in the V3 loop conferred CXCR4-use; however, the loss of macrophage-tropism by one variant was due to a single mutation in the start codon of vpu. We discuss how V3 loop and vpu mutations acquired by replication in T-cell lines in vitro correlate with similar changes reported for primary isolates and HIV-1 sequences in vivo.

Non-syncytium-inducing (NSI) human immunodeficiency virus type 1 (HIV-1) strains that use CCR5 as a co-receptor are predominantly transmitted and persist throughout infection. In contrast, syncytium-inducing (SI) CXCR4-using viruses are transmitted inefficiently yet these viruses emerge in about 50% of AIDS patients late in disease (Asjo et al., 1986; Connor & Ho, 1994; Connor et al., 1997; Scarlatti et al., 1997; Tersmette et al., 1988). The selective pressures that drive or prevent changes in co-receptor use are still poorly understood. Generally, the acquisition of basic amino acids at specific sites in the V3 loop is associated with syncytia formation and CXCR4-use (De Jong et al., 1992; Fouchier et al., 1992; Shioda et al., 1991). In vitro, R5 strains can be adapted to use CXCR4 by repeated passage in CD4+ T-cell lines that do not usually express CCR5 (Harrowe & Cheng-Mayer, 1995; Klasse et al., 1996). CXCR4-use has been associated with the loss of macrophage-tropism (Di Marzio et al., 1998), although this is controversial (Simmons et al., 1996, 1998; Stent et al., 1997; Verani et al., 1998; Yi et al., 1998). Here, we assess co-receptors and tropism of two HIV-1 SF162 variants selected for replication in C8166 cells. Both variants could use CCR5 and CXCR4 as co-receptors, yet only one was macrophage-tropic.

SF162 was first selected for replication in the MOLT4 T-cell line as previously described (Simmons et al., 1995). MOLT4 cells express low amounts of CCR5 on the cell surface that are undetectable by flow cytometry yet the cells support infection by a subset of primary R5 HIV-1 isolates (Dejucq et al., 1999). SF162 derived from MOLT4 cells retains the macrophage-tropic R5 phenotype of the parental strain and fails to replicate in the majority of T-cell lines including C8166 cells (Simmons et al., 1995). SF162-infected MOLT4 cells were irradiated (80 Gy) and co-cultivated with C8166 cells. After 2–3 weeks culture, large syncytia were observed. Supernatant taken from these cells was used to infect fresh C8166 cells and to produce virus stocks. Two independently derived variants that induced large syncytia after infection of C8166 cells were isolated. The two variants, along with wt SF162, were titrated on C8166 cells, primary macrophages and phytahaemagglutinin (PHA), IL-2-stimulated peripheral blood mononuclear cells (PBMCs). Macrophages and PBMC cultures were prepared as previously described (Simmons et al., 1996). One variant, SF162-D, retained the ability to replicate in macrophages and PBMCs and was therefore dual-tropic. However, the second variant, SF162-T, was T-cell line-tropic and replicated efficiently in several T-cell lines including C8166 cells as well as MOLT4 and SUP71 cells (data not shown), but poorly in PBMCs and barely at all in macrophages (Table 1).

To establish the genetic basis for the differences in the biological phenotypes observed for the two variants, DNA was extracted from C8166 cells infected with either SF162-D or SF162-T and 3’ sequences containing the complete envelope gene were amplified using primers PE1, 5’ AAGCATCCAG GAATGCAGCTTAAG 3’ (SF162 nt 121–144), and BE6, 5’ TTTTTCCAAGAGTGAGATACTGC 3’ (SF162 nt 3149–3126). PCR products were then directly cloned into pGEM-T (Invitrogen). BsuI and XhoI enzymes were used to excise 2·6 kb fragments containing the entire envelope sequence. These sequences were subcloned into the 3’ half of SF162 substituting...
for the equivalent wt sequence (Fig. 1a). A 476 bp region upstream of \textit{env} was investigated by amplifying sequences between the \textit{EcoRI} and \textit{BstI} sites using primers \textit{vp}1, 5' GACACTAGAAGCTTTAGAGGAGCT 3' (based on HXB2 sequence nt 5158–5181), and \textit{vp}2, 5' CAGCATACTAGATCTACAAATCC 3' (SF162 nt 566–546). This region encodes the 3' end of \textit{vpr}, the 5' coding regions of \textit{tat} and \textit{rev} as well as 155 of 243 \textit{vpu} amino acids (Fig. 1a).

Sequence analysis revealed that the T- and D-tropic strains had two amino acid substitutions (I → R and A → V) in the V3 loop (Fig. 1b). Few other amino acid changes were observed outside the variable regions of \textit{env} and most were common to both variant strains (data not shown). Moreover, a single base mutation was observed in the translation initiation codon of \textit{vpr} (ATG → ATA) of the T-tropic variant as compared to wt and D-tropic SF162 (Fig. 1b). This mutation was the only nucleotide change observed in the \textit{EcoRI–BstI} region.

The respective importance of the \textit{vpu} and \textit{env} mutations in the observed phenotypes was examined by constructing four chimeric molecular clones (Fig. 1c). In particular, the \textit{vpu} mutation was investigated by cloning the \textit{EcoRI–BstI} fragment into the 3' half of SF162, together with the envelope of the T-tropic strain (TT) or the D-tropic strain (TD) as \textit{BstI–XhoI} fragments (Fig. 1c). Corresponding clones containing wt SF162 \textit{vpu} with either the T-tropic (wT) or D-tropic (wD) envelopes were also constructed. The 3' halves of the constructed chimeric and wt SF162 genomes were excised from the pUC19 plasmid by \textit{EcoRI} digestion and ligated to a similarly excised fragment containing the 5' half of the genome. Virus stocks were prepared in C8166 cells transfected by electroporation with the ligated full-length SF162 constructs and assayed for reverse transcriptase (RT) activity using a Lenti-RT activity kit (Cavidi Tech).

We determined whether SF162 D- and T-tropic variants, and the chimeric viruses (see above) used different co-receptors as compared to wt SF162. The human glioma cell line (U87/CD4) as well as other cell lines expressing human CD4 with either CCR5 or CXCR4 were tested for sensitivity to wt SF162 and variant infection. While wt SF162 used only CCR5, both the dual-tropic and T-tropic variants as well as the four molecular clones used both CCR5 and CXCR4 to infect U87/CD4 cells (data not shown). We also tested alternative co-receptors such as GPR15/BOB, STRL33/BONZO (on GHOST cells), CCR8 (on CCC/CD4 cells), and CCR1, CCR2b and CCR3 (on U87/CD4 cells) were exploited by the SF162 variants. Only CCR8 was used by both variant viruses as well as wt SF162 (data not shown).

We next investigated macrophage-tropism of the four chimeric molecular clones TT, TD, wT or wD to ascertain the contribution of the \textit{vpr} and envelope mutations on the different tropism phenotypes observed for the uncloned variants. We infected three macrophage cultures derived from different donors with equivalent infectious doses (measured on U87/CD4/CXCR4) of the chimeric molecular clones. Virus replication in macrophages was monitored by assessing RT activity in supernatants collected every 6–8 days for up to 25 days. Both wD and wT molecular clones productively infected macrophages (Fig. 2a); however, replication by the TD and TT clones was severely impaired. The TT clone differs from wT (and TD differs from wD) by only a single mutation that disrupts the start codon of \textit{vpu}. Thus, this \textit{vpu} mutation alone abrogates replication in macrophages by the TT and TD SF162 chimeric viruses.

The infectivity titres of SF162 wt, variants and chimeric viruses assessed in macrophages and PBMCs were also compared. Fig. 2(b) shows macrophage:PBMC infectivity ratios plotted for each of these viruses. Low ratios resulting from significantly lower infectivity for macrophages as compared to PBMCs were noted for all viruses containing a mutated \textit{vpu}. These results confirm that the \textit{vpu} mutation alone results in severely reduced virus replication in macrophages but also highlights the fact that the loss of Vpu consistently affects macrophage-tropism more severely than replication in PBMC.

In vivo, co-receptor switching for R5 strains to CXCR4-use is a relatively rare event, with only about half of AIDS patients carrying SI strains. Yet in vitro, it is often simple to elicit a co-receptor switch to CXCR4 by adapting a CCR5-using virus for replication in a CD4+ CXCR4+ T-cell line. We suspect that some strains need few amino acid substitutions to acquire CXCR4-use since they readily adapt, while others need more extensive passage and presumably require multiple changes. The in vivo pressures that select for or against CXCR4-use are currently unclear, although immune mechanisms such as neutralizing antibodies (Lathe et al., 1997) and non-immune mechanisms such as SDF-1 blockade and/or downregulation

### Table 1. Cell tropism of SF162- and C8166-derived variants

<table>
<thead>
<tr>
<th>Virus</th>
<th>Cell source</th>
<th>PBMC (TCID&lt;sub&gt;50/ml&lt;/sub&gt;)</th>
<th>C8166 (TCID&lt;sub&gt;50/ml&lt;/sub&gt;)</th>
<th>Macrophage (f.f.u./ml)</th>
</tr>
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<tbody>
<tr>
<td>SF162</td>
<td>PBMC</td>
<td>1.2 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>20</td>
<td>5.9 × 10&lt;sup&gt;4&lt;/sup&gt;/9.9 × 10&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>SF162-D</td>
<td>C8166</td>
<td>6.3 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>9.3 × 10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>8.2 × 10&lt;sup&gt;4&lt;/sup&gt;/2.0 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>SF162-T</td>
<td>C8166</td>
<td>630</td>
<td>6.3 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>1.6 × 10&lt;sup&gt;4&lt;/sup&gt;/1.6 × 10&lt;sup&gt;3&lt;/sup&gt;</td>
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Fig. 1. SF162 PCR, cloning strategy and mutations. (a) Schematic outline of the HIV-1 genome with restriction sites used to clone SF162 variant sequences. (b) Sequence of vpu and V3 loop crown for wt SF162, T- and D-tropic mutants. Base changes as compared to wt SF162 are indicated by asterisks. (c) Construction and schematic representation of the genomic organization of SF162 chimeric viruses and recombinant molecular clones. Chimeric molecular clones carrying mutated Vpu from the T-tropic variant were associated with the env determinant of the T-tropic (TT) or D-tropic variant (TD). Similarly, wt vpu from SF162 was combined with env from T-tropic (wT) or D-tropic mutants (wD).

of CXCR4 (Agace et al., 2000; Fauci, 1996; Pablos et al., 1999) have been suggested (Michael & Moore, 1999). Whatever the selective pressure, it appears to wane late in the disease when X4 strains emerge and persist. In vivo, CXCR4-use is associated with acquisition of positively charged amino acids at one (or both) of two specific sites in the V3 loop (CTR PNNNTR
KRI GPGRA FYATG KIIGN IRQAH C) (De Jong et al., 1992; Fouchier et al., 1992; Shioda et al., 1991), although this correlation was not observed for Romanian subgroup F isolates (Holm-Hansen et al., 1995). CCR5-using viruses adapted in vitro to use CXCR4 on CD4+ T-cell lines may also acquire positively charged amino acids at the same V3 sites, for example, the 17.11 variant of JRCSF (Klasse et al., 1996). The SF162 variants described here, however, gain a positively charged amino acid (I→R) at a distinct V3 site (ITRGPG). Positively charged amino acids are present only rarely at this position in V3 loops catalogued in the HIV sequence database (http://hiv-web.lanl.gov/). Some strains contain a similar motif to the SF162 variants described here (e.g. ITRGPGR, sequence V3221), while others have a two base insertion that confers a positively charged amino acid adjacent to the GPGR motif (e.g. IRIQRGPGR, sequence V3166). It is unclear whether these database sequences represent CXCR4-using viruses. However, this sequence location may be a rare site for positively charged residues that confer the use of CXCR4 to R5 strains in vivo. Different selection pressures present during T-cell line adaptation in vitro may allow the V3 loop to adopt distinct conformations that are rare and selected against in vivo, but which may allow positively charged amino acids at distinct V3 sites to confer CXCR4-use.

Cheng-Mayer’s group also described an SF162 variant (R3H) selected independently for replication in the HUT78 T-cell line (Harrowe & Cheng-Mayer, 1995). Remarkably, the R3H strain contained the same two V3 loop amino acid substitutions as the variants described here, of which the I→R substitution was shown to confer both CCR5- and CXCR4-use on cell lines. Yet in contrast to our D-tropic variant and chimeric viruses, Harrowe’s group reported that neither R3H nor an SF162 mutant with only the two V3 loop changes replicated in primary macrophages (Harrowe & Cheng-Mayer, 1995). Macrophage cultures prepared in different laboratories have distinct sensitivities to CXCR4-using HIV strains, a phenomenon that has resulted in controversy over whether X4 viruses can infect macrophages per se. Our results, however, confirm that neither T-cell line adaptation nor CXCR4-use necessarily precludes macrophage-tropism.

Several reports have already shown the importance of Vpu for virus replication in macrophages (Balliet et al., 1994; Kawamura et al., 1994; Schubert et al., 1995), yet defective vpu genes are present in vivo. Nearly 5% of vpu sequences (derived from primary isolates or directly from patients) in the HIV sequence database contain a mutated ATG translation start codon. Moreover, several primary HIV-1 strains including YU2 and AD8 strains (Schubert et al., 1999) contain mutations in the vpu ATG codon, yet replicate efficiently in primary macrophages. These strains appear to have overcome the requirement of Vpu for virus replication in macrophages by an adaptation that involves determinants in the envelope glycoprotein (Schubert et al., 1999). For most HIV-1 strains, the function of Vpu for virus replication in macrophages is unclear. Vpu has two domains responsible for distinct functions. The N terminus of Vpu forms a transmembrane region that has been implicated as an ion channel and is important for the regulation of virus release (Schubert et al., 1996), whereas the cytoplasmic C-terminal part of Vpu interacts with CD4 and redirects it via the ubiquitin pathway into proteosomes for degradation (Schubert et al., 1998). Thus both Vpu functions may aid efficient release of virions (Lama et al., 1999), a property that seems essential for most HIV-1 strains to replicate in macrophages.
In summary, HIV-1 R5 viruses adapting to replicate in T-cell lines acquire multiple mutations in several genes. Amino acid substitutions in the envelope V3 loop confer the use of CXCR4, which allows entry into T-cell lines. The loss of macrophage-tropism observed for many T-cell line adapted HIV-1 strains, however, can be caused by mutations that abrogate accessory gene functions such as vpu.

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