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Selection following isolation of human immunodeficiency virus type 1 in peripheral blood mononuclear cells and herpesvirus saimiri-transformed T cells is comparable

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In attempts to improve isolation rates and virus yields for human immunodeficiency virus (HIV), the use of herpesvirus saimiri-immortalized T cells (HVS T cells) has been investigated as an alternative to/improvement over peripheral blood mononuclear cells (PBMCs). Here we characterize isolates rescued, in the two cell types, from two asymptomatic, long-term non-progressing HIV-1-infected individuals. All rescued viruses replicated in PBMCs and HVS T cells only, displaying a non-syncytium inducing (NSI) phenotype, and using CCR5 as co-receptor. Furthemore, PBMC/HVS T cell virus pairs displayed similar neutralization profiles. Full-length, expression-competent *env* genes were rescued from all virus isolates and directly from the patient samples using proviral DNA and viral RNA as templates. Compared with the sequences retrieved directly from the patient samples, both cell types showed similar selection characteristics. Whilst the selections were distinct for individual patient samples, they shared a common characteristic in selecting for viruses with increased negative charge across the V2 domain of the viral glycoproteins. The latter was observed at the *env* gene sequencing level for three other patients whose HIV strains were isolated in PBMCs only. This further supports a common selection for viral sequences that display a macrophage-tropic/NSI phenotype and shows that HVS T cells are a viable alternative to PBMCs for HIV-1 isolation.

Introduction

Isolation and characterization of human immunodeficiency virus (HIV) isolates from asymptomatic patients, particularly those classified as long-term non-progressors (LTNP), may provide important information relating to virus pathogenesis and thereby facilitate vaccine and antiviral development. In terms of growth kinetics, cell tropism and co-receptor usage, HIV type 1 (HIV-1) can be classified into two main groups: slow-growth, macrophage (M)-tropic R5 virus and fast-growth, T cell (T)-tropic X4 virus (Deng *et al.*, 1996; Dragic *et al.*, 1996; Feng *et al.*, 1996). The majority of viruses isolated from asymptomatic infected individuals are M-tropic and of the NSI phenotype (Schuitemaker *et al.*, 1992; Connor & Ho, 1994). However, isolation rates of such viruses in mitogen- or cytokine-stimulated PBMCs can be poor and low virus titres have often been reported (Fenyo *et al.*, 1988). Additionally, there is inconsistent replication of these HIV strains in different donor PBMCs (Evans *et al.*, 1987; Spira & Ho, 1995). Hence, there is need for an improved culture-isolation system.

We have reported isolation of HIV-1 from LTNP, at high titre, in the herpesvirus saimiri-immortalized T cell (HVS T cell) line, CN-2 (Vella *et al.*, 1999a). CN-2 was immortalized from CD8-depleted, HIV-seronegative donor PBMCs and shown to express CD4, CD45RO, CD26, CD7, CCR5 and CXCR4 at the cell surface, reminiscent of activated memory T cells, which are targeted by HIV-1. However, several studies have reported the loss of genetic polymorphism present in HIV-1 quasispecies after isolation in T cell lines and PBMCs and the phrase 'to

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The EMBL accession numbers for the 63 sequences reported in this paper are AJ418478–AJ418540 (in the order presented in Fig. 3).

isolate is to disturb' has been used (Meyerhans *et al.*, 1989; von Briesen *et al.*, 1990; Kusumi *et al.*, 1992). Alterations in biological characteristics, such as sensitivity to neutralizing antibodies between viruses grown in PBMCs and those grown in macrophages or T cell lines, have also been reported (Sawyer *et al.*, 1994; Willey *et al.*, 1996). To address such problems in relation to HIV-1 isolation in HVS T cells, viruses isolated from two patients in PBMCs and four HVS T cell lines were characterized phenotypically.

For HIV-1 genotypic analyses the *env* gene was selected as it encodes glycoproteins that are crucial for virus entry and are major determinants of cell tropism (reviewed in Douglas *et al.*, 1997). Binding sites for host cell receptors reside on gp120 whilst membrane fusion components are contained in gp41. In addition, the glycoproteins are major targets of the host immune response and the only ones to which virus-neutralizing antibodies are raised. Complete *env* genes were amplified and cloned directly from patient DNA and plasma RNA extracts and compared with those in viruses produced by their respective culture isolates. Additionally, this approach supplied information on three HIV-1 regulatory genes (*vpu*, *tat* and *rev*), the translation products of which can have profound effects on virus replication.

PBMCs and HVS T cells were shown to have similar properties by selecting for viruses that would replicate in these two cell types only, with an NSI phenotype, and individual PBMC/HVS T cell virus pairs had similar neutralization profiles. Sequencing studies showed that both cell types selected similar variants from the virus quasispecies and, whilst selection was specific for individual patient/virus samples, all virus isolates displayed the common characteristic of carrying increased negative charge across their glycoprotein V2 domains compared with sequences derived directly from the patient samples. These results indicate that HVS T cells are a viable alternative to PBMCs for the isolation of HIV-1.

Methods

■ **Cells.** PBMCs were isolated by Ficoll gradient centrifugation of whole blood, taken 22 July 1997, from five HIV-1-infected individuals enrolled in the Chelsea & Westminster (CW) long-term infected cohort. All patients were male, had been infected for over 10 years and were designated as non-progressors (NP: CW002, CW010, CW012, CW037 and CW048) based on maintenance of $CD4^+$ cell counts above 500/μl (666, 675, 674, 899 and 821, respectively, at or near the time of sampling). One patient (CW002) had received drug treatment, nelfinavir.

A panel of 11 cell lines were used in cell tropism studies. These were the T cell lines MT-2, PM-1, JM, H9, C8166, Molt-4-clone8, Jurkat, Jurkat-tatIII and CEM-SS, the monocytoid line U937 and the human myeloid leukaemia cell line HL60. The HVS T cell lines CN-1 and CN-2 (Vella *et al.*, 1999a), AC (Vella *et al.*, 1997) and CP-1 (Vella *et al.*, 1999b) were used in virus isolation studies. The CD8-depleted, HIV-1 seronegative, donor PBMC pool has been described (Vella *et al.*, 1999b). CN-1 and CN-2 came from the same blood donation, provided by an HIV-1 seronegative donor (HLA class II haplotype: DRB1*0401, DRB1*0701, DRB4*0103, DRB4*0101, DQB1*0303, DQB1*0301), which had been CD8-depleted and, for CN-1, enriched for CD45RO and CD7 by FACS sorting, prior to immortalization. Likewise, CP-1 was immortalized from the CD8-depleted donor PBMC pool used for isolation. Cells were cultured as described previously (Vella *et al.*, 1999a, b).

Mega-glioblastoma U87 cells stably expressing human CD4 and one human chemokine receptor (CCR1, CCR2b, CCR3, CXCR4 or CCR5) were employed in co-receptor usage assays (Bjorndal *et al.*, 1997). The cells were grown in DMEM (Gibco) containing 15% FBS with 300 μg/ml G418. To select for co-receptor expression, cultures were supplemented with 1 μg/ml puromycin.

■ **Antibodies.** Human plasma/sera and a panel of monoclonal antibodies (mAbs) were used to compare the sensitivity of PBMC and HVS T cell virus isolates to neutralization. Plasma samples were obtained from five LTNP (CW010, CW012, CW031, CW037 and CW082; Vella *et al.*, 1999a). V3 loop-binding mAbs were ARP3023 and ARP3056 and those recognizing the CD4 binding site were ARP388/389, ARP3054 and ARP3065.

■ **Virus isolation and titration.** Freshly prepared patient PBMCs were co-cultivated with equal numbers of HIV-1 seronegative donor PBMCs that had been stimulated with PHA for 3–4 days. Subsequently, patient PBMCs (CW010 and CW012), which had been stored in liquid nitrogen for 3 months, were co-cultivated with equal numbers of HVS T cells. Production of virus was monitored with commercial p24-ELISA kits (Abbott). Samples containing the peak p24 concentrations were used to determine the biological and genotypic phenotypes of the rescued viruses. The 50% tissue culture infective dose ($TCID_{50}$) of primary isolates rescued in either PBMCs or HVS T cells was determined on the freshly stimulated PBMC pool as described (Vella *et al.*, 1999b).

■ **Virus neutralization assay.** The neutralization sensitivity of PBMC and CN-2 isolates was compared in both PBMCs and CN-2 using 7 day microneutralization assays with a constant virus challenge of 100 $TCID_{50}$ against 1:5 dilutions of heat-inactivated serum/plasma. mAbs were used at a concentration of either 5 μg/ml (ARP3023, ARP388/389) or 10 μg/ml (ARP3056, ARP3065). For the assay, 50 μl aliquots of virus were mixed with 50 μl plasma/serum or mAb in duplicate in 96-well flat-bottomed microtitre plates and incubated at room temperature for 1 h. Freshly stimulated pooled PBMCs or CN-2 cells in exponential phase growth were then added (4×10^4 cells in 100 μl) and incubated overnight. Residual p24/Ab was removed with extensive washes, and plates incubated for a further 6 days, after which virus production was measured by p24-ELISA. Virus controls (virus plus cells alone), cell controls (cells plus antibody alone) and neutralization of tissue-culture-adapted strains of HIV-1, GB8 and JRFL, were included. The neutralization titre (% inhibition of p24 production) was determined at 7 days post-infection (p.i.) using the formula: % inhibition = mean OD of virus controls − (mean OD of virus + Abs)/mean OD of virus controls × 100.

Neutralization by serum/plasma and mAbs (at the concentrations used) was considered positive when an inhibition of 70% or greater was observed.

■ **Cell tropism and co-receptor usage.** The appropriate T cell line and PHA-stimulated PBMCs (5×10^3 cells each) were inoculated in duplicate with 50 μl of virus-containing culture supernatant, in 96-well round-bottomed plates, and incubated for 3 h at 37 °C. After washing three times with RPMI, the cells were resuspended in 200 μl of culture medium. Half of the culture supernatant was removed and replaced with fresh medium as required and syncytium formation was observed daily for 14 days. Supernatant was tested by p24-ELISA at 14 days p.i.

To determine the co-receptor usage of the viruses, 10^4 cells of each $CD4^+$ U87 cell line expressing an HIV-1 co-receptor were inoculated with 200 $TCID_{50}$ of virus (determined on PBMCs) and screened for infection as described (Dittmar *et al.*, 1997).

Table 1. Oligonucleotide primers used for RT–PCR, PCR and sequencing

HIV-specific primers are related to their position (HXB2 no.) in the HIV-1_{HXB2} genome.

	Sequence	HXB2 no.
RT–PCR, PCR		
NEFR	GTCATWGGYCTYARAGGTACCTGDGG	9009–9035
TATF	GAGCCCTGGAASCAYCCRGGRAGTCAGCC	5854–5882
FENV	TAAGAGCTCGAGCAGAAGAYAGTGGCAATGARAGYGA	6199–6235
RENV	ACCACAGAATTCTTTGACCAYTTGCCACCCATBTTA	8792–8817
Sequencing, forward primer		
Q7F*	TCGAGCCCGCGGTGATCATCCGGATATCG	
M2A	TGGGCCACACATGCCTGTGTACC	6428–6450
M5	GTCAGCACAGTACAATGTACA	6947–6967
M7R	TGTRGAGGRGAATTTTTCTAYTG	7355–7377
M11R	GGGATKTGGGGYTGCTCTGG	8003–8022
M22R	GTTAGGCAGGGATAYTCACC	8345–8364
Sequencing, reverse primer		
SEQ1	GGGGTTAATTTTACACATGG	6575–6594
V3-1R	TTCTGCTAGRCTRCCATT	7007–7024
V3-3R	TAGAAAAATTCYCCTCYACARTTAA	7350–7375
R20R	TTGAGGRYTTCCCACCCC	8587–8604
R22N	GTTTCCAGAGCAACCCA	8009–8027
Q7R*	AATTCGATATCCGGATGATCACCGCGGC	

* Primers Q7F and Q7R are located in the pQ7 vector.

■ **Rescue of viral *env* genes.** *Env* genes were rescued from DNA extracts of uncultured patient PBMCs, and RNA extracts of plasma and culture supernatants. Viral RNAs were extracted from 100 µl of plasma or peak titre culture supernatants using the SDS–proteinase K lysis/phenol/chloroform method and cDNA was synthesized using Primer NEFR (Table 1). For DNA and cDNA templates, two rounds of PCR were performed using primer pairs TATF/NEFR and FENV/RENV (Table 1; Douglas *et al.*, 1996). Full-length *env* genes, with terminal *Xho*I and *Eco*RI sites, were cloned into the expression vector, pQ7. Clones that expressed full-length gp160, detected by Western blot, were selected for sequencing with a panel of primers (Table 1). ABI 377 sequencers were used with Big-Dye kits and sequences assembled using gap-4 (Bonfield *et al.*, 1995).

■ **Phylogenetic and translation product analyses.** Nucleotide sequences were imported to the Genetic Data Environment (GDE) for manipulation (Smith *et al.*, 1994). Translation products were aligned using CLUSTAL V, and the programme 'variate' (Douglas *et al.*, 1997) was used to analyse the physico-chemical properties of the glycoprotein groups. The glycoprotein alignments were used to produce nucleotide alignments that were used for phylogenetic analyses, based on the neighbour-joining method (Pearson *et al.*, 1999).

Results

Virus isolation

Isolation in the CD8-depleted PBMC pool was performed with freshly purified patient cells. For patients CW010 and CW012, virus isolation was attempted in HVS T cells AC, CP-1, CN-1 and CN-2 using patient cells that had been stored in liquid nitrogen for 3 months. All co-cultures except CW010/AC were isolation-positive. Virus titres, based on p24 production, ranged from 2·2 ng/ml to 4 µg/ml (Fig. 1). p24 production was first detected in PBMC cultures, as early as 4 days after co-culture, and reached high concentration (20–240 µg/ml) within 7–14 days. For HVS T cell culture, p24 production was detected after 14–20 days and peak p24 production (3–4000 µg/ml) occurred around 30 days after co-culture. This slower isolation in HVS T cells appears to be an inherent property of the cells as it is seen consistently, whether or not the patient's cells have been frozen (Vella *et al.*, 1999a). However, both CN-2 and CP-1 cells produced higher virus titres than PBMCs and only cultures of CW010 in CN-1 and AC gave lower virus titres than those seen in PBMCs. The high-level p24 production in HVS T cell co-cultures was maintained for 2 months without the need to add fresh cells (data not shown).

Cell tropism study

The question of selection pressures in HVS T cells compared with PBMCs was addressed by inoculation of MT-2, C8166, PM1, U937, H9, JM and PBMCs with approximately 700 pg p24 cell-free tissue culture supernatants for all isolates from CW010 and CW012. Isolates could be propagated in PBMCs only, as determined by p24-ELISA at 14 days p.i., and showed an NSI phenotype. The cell tropisms of PBMC and CN-2 isolates from CW010 and CW012 were studied further following passage of PBMC isolates in CN-2 and CN-2 isolates in PBMCs for 14 days. Culture supernatants containing 250 ng

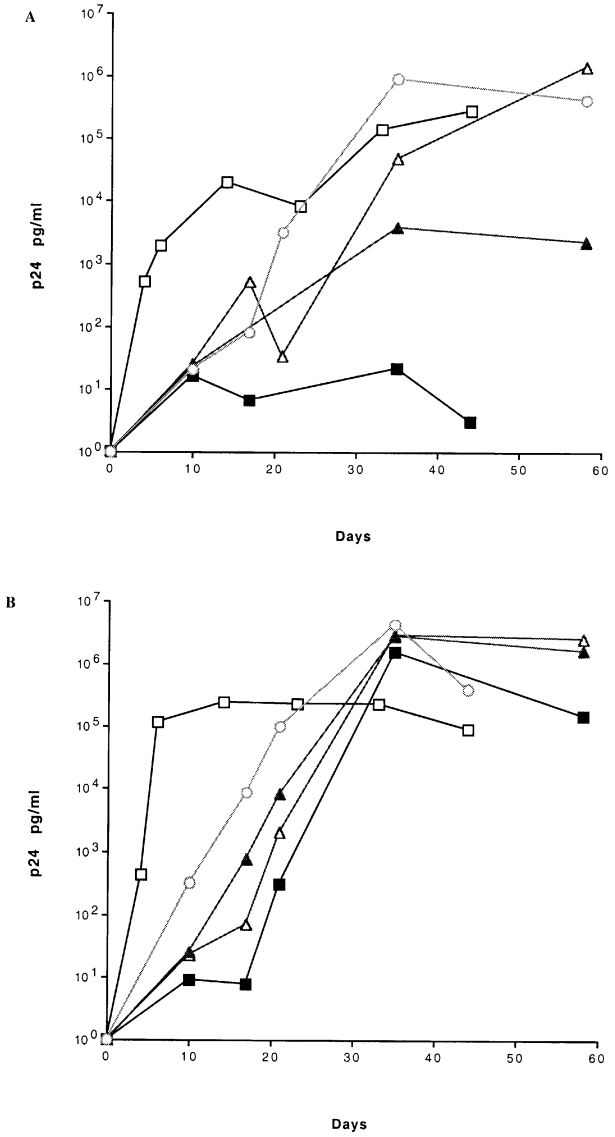

Fig. 1. Virus isolation in PBMC and HVS T cell lines. (A) CW010. (B) CW012. Virus production in co-culture with PBMCs (□) or HVS T cell lines AC (■); CP-1 (△); CN-1 (▲); CN-2 (○).

p24 were then inoculated in quadruplicate on to the panel of 11 cell lines. Syncytia were not seen and p24 production was not detected, showing the lack of growth of CN-2/PBMC isolates in cell lines to be stable on passage.

Co-receptor usage assays showed that all isolates from CW012 infected U87/CD4$^+$/CCR5$^+$ cells only, confirming their R5 status, while isolates from CW010 in addition infected U87/CD4$^+$/CCR3$^+$ cells showing that they were of R5R3 phenotype. These results are in agreement with the NSI phenotypes of the viruses.

Sensitivity to neutralization

A panel of mAbs, autologous and heterologous plasma were tested against paired PBMC and CN-2 virus isolates (Table 2). Although small differences in sensitivity between viruses isolated from the same patient were seen, the neutralization profiles were similar using 70% inhibition of p24 production as compared with the virus control as the cut-off. Plasma C012 neutralized its own isolates whilst plasma C010 did not. Only plasma C082 neutralized virus from CW010. Plasmas C031 and C037 neutralized virus from patient CW012. Neither anti-V3 nor anti-CD4 binding site mAbs neutralized virus from patients CW010 and CW012.

Sequence analysis

To determine the potential selection effects of cell culture on virus isolation, full-length *env* genes of uncultured proviruses (from patient PBMCs), cell-free virus (from plasma) and 12 isolates (cell-free tissue culture supernatants) were cloned. In total, 17 clones (five DNA, three plasma-derived, two PBMC, three CP-1, two CN-1 and two CN-2) were sequenced for CW010 and 19 clones (four DNA, two plasma-derived, three PBMC, two AC, two CP-1, two CN-1 and four CN-2) for CW012. For CW002, CW037 and CW048, three clones each were sequenced from PBMC isolates and four to seven from patient DNA/plasma.

Phylogenetic analysis was performed on sequences generated here together with those of reference strains in the Los Alamos HIV Database (http://hiv-web.lanl.gov). The nucleotide alignment spanned 2772 positions, encompassing entire *env* genes and retaining gaps introduced to improve the alignment (predominantly in regions encoding gp120 variable domains). A thousand trees were calculated and a consensus tree generated. All CW-derived viruses belonged to HIV-1 subtype B and individual patient *env* gene clones grouped in distinct clusters with 100% bootstrap values at their origins, indicative of no cross-contamination during virus isolation and *env* gene cloning (Fig. 2). Whilst phylogenetic distances were small, HIV quasispecies were apparent in all five patients and there was some suggestion of disparity between provirus- and virus-derived clones, notably for patients CW002 and CW048, though this may reflect the relatively low number of clones sequenced from each source. For all five patients, virus isolation in PBMCs (clones A; Fig. 2) selected for *env* genes that were distinct from those present *in vivo* (proviral DNA and plasma RNA, clones D and P; Fig. 2). Similarly, for CW010 and CW012, where virus isolation in HVS T cells was attempted, recovered *env* genes were most closely related to those derived from their respective PBMC isolates, with one exception (CW012H-533), which had a plasma-derived sequence (CW012P-002) as its closest relative. Overall, this suggests selection of relatively minor HIV variants from the *in vivo* quasispecies during virus isolation by the cell co-culture routes.

HIV glycoproteins are major determinants of tissue tropism due to their receptor-binding and membrane fusion functions and, therefore, selection that can occur during virus isolation. As virus isolation routinely employs cell co-culture, selective forces may favour isolation of viruses with good cell–cell

Table 2. Neutralization of PBMC and CN-2 isolates by autologous and heterologous plasma and monoclonal antibodies

Percentage inhibition of p24 production after treatment with antibody compared with virus controls (no antibody) are shown. Values are the mean of duplicate assays and those greater than 70%, considered positive for virus neutralization, are shown in bold type. Laboratory strains GB8 and JRFL were included as controls. ND, Not done.

		Plasma					Monoclonal antibodies			
		Autologous		Heterologous			V3 loop		CD4 binding site	
Patient	Virus	010	012	082	031	037	ARP3023	ARP3056	ARP3065	ARP388/389
CW010	PBMC isolate	54	ND	**74**	65	37	41	43	55	0
	CN-2 isolate	49	ND	**78**	58	43	26	33	50	0
CW012	PBMC isolate	ND	**79**	42	**71**	**78**	14	35	54	23
	CN-2 isolate	ND	**83**	39	**70**	**83**	33	41	44	3
Controls	GB8	ND	ND	**91**	**92**	41	ND	ND	ND	ND
	JRFL	ND	ND	ND	ND	ND	40	33	63	43

fusion characteristics over those with good virus–cell fusion properties. However, all isolations made here yielded viruses with an NSI phenotype. To investigate this further, translation products of rescued *env* gene clones were aligned to allow inspection of amino acid differences between clones derived from *in vivo* sampling (DNA and RNA) and culture isolates (RNA; data not shown). For all five patients, though it was recognized that the sampling was limited, there was evidence for selection following isolation in PBMCs of minor/undetected variants in the *in vivo* sampling. The selections differed in terms of specific amino acids and domains involved for each patient, possibly reflecting the diversity of the virus quasispecies in individual patients. For individual patients, the HIV-1 glycoprotein diversity was 5·0–10·8% in the *in vivo* situation with CW048 showing the lowest diversity (CW048 < CW002 < CW037 < CW012 < CW010). In each case, when glycoprotein sequences derived from virus isolates were considered as well, this diversity was increased to 6·1–12·8% with CW048 again showing the lowest diversity (CW048 < CW037 < CW012 < CW002 < CW010).

HVS T cell isolation was performed for patients CW010 and CW012 and glycoprotein sequences recovered related most closely to those seen in their respective PBMC isolates (Fig. 2), which suggests that selection pressures are similar in activated HIV-1-seronegative donor PBMCs and HVS T cells. Overall, intra-patient glycoprotein homologies, for *in vivo* sampling and culture isolates, fell in the range 87·2–99·9%, whilst inter-patient homologies were 82·4–87·0% (not shown). CW010-derived sequences showed the greatest inter-group diversity throughout the glycoprotein and they were the only set of sequences to carry inter-group sequence substitutions at positions that have been shown to affect CCR5 binding (data not shown). This may relate to the R5R3 phenotype of CW010 isolates, as the amino acid substitutions observed would be expected to reduce binding to CCR5 (Rizzuto *et al*., 1998). In addition, whilst most *in vivo* clones had the typical GPGR motif at the tip of the V3 loop (LaRosa *et al*., 1990), CW010D-005 and all culture isolate clones contained a GPGS motif, which would result in a loss of positive charge (Fig. 3a). The presence of positive charge on V3 loops, notably at positions 9 and 27 in our alignment, has been associated with the ability of HIV-1 to induce syncytia (Fouchier *et al*., 1992). On this basis, all clones derived from CW010 isolates were predicted to have an NSI phenotype, whilst three of eight *in vivo*-derived clones (CW010D-007, CW010P-007 and CW010P-008), carrying positively charged residues in these two positions, might be derived from SI viruses. The isolate sequences were most closely related to an *in vivo*-derived DNA sequence (CW010D-005), confirming phylogenetic results, though sequence variation in the cytoplasmic tail of gp41 was significant (data not shown), possibly indicating the presence of *env* gene recombinants in the quasispecies.

Sequences derived from patient CW012 showed slightly higher homologies than those derived from CW010. The most notable variation between *in vivo* and isolate sequences was that clones derived from culture isolates generally had longer V2 domains (Fig. 3b). The majority of clones had His at position 9 and Asp at position 27 of the V3 loop, suggesting an NSI phenotype for all these clones (Fig. 3a). Overall there were no significant differences in the number of *N*-linked glycosylation sequons between the cloned glycoprotein products.

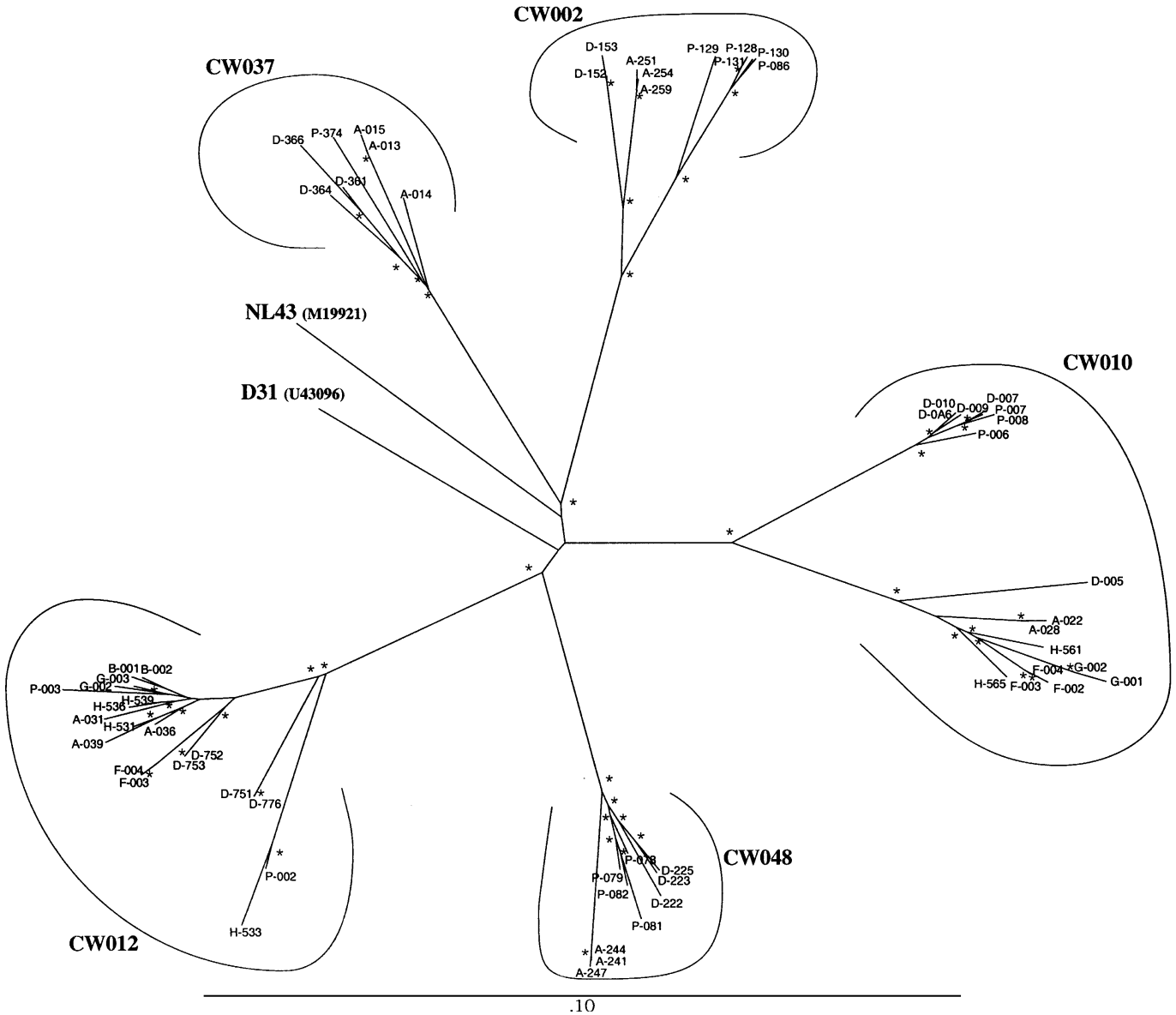

Fig. 2. Phylogenetic relationships of rescued *env* genes. A representative phylogenetic tree for full-length *env* genes of two subtype B reference strains and patients' uncultured proviruses, cell-free viruses and culture isolates is shown. Clones are grouped by their CW number (002, 010, 012, 037 and 048) and labelled with suffixes indicating their source [D, patient cells (DNA); P, plasma (RNA); A, PBMC isolates; H, CN-2 isolates; G, CN-1 isolates; F, CP-1 isolates; B, AC isolates] and individual clone number. The accession numbers of the two reference strains are given in parentheses. Nodes where bootstrap values are greater than 70% (from a consensus tree of 1000 calculations) are marked (*). The phylogenetic distance bar is shown below the tree.

a) V3 domain

```
           1         11        21
           |         |         |
    NL43_1 NNNTRKSIRIQRGPGRAFVTIG-KIGNMRQ

CW010D-010 NNNTRKSIPI--GPGRALYATGDIKIEIRQ
CW010D-007 ......G.H.................K...
CW010D-009 ..............................
CW010D-0A6 ..............................
CW010D-005 ........SF.....S.M....A...D...
CW010P-006 ........HV....................
CW010P-007 ......G.H.................K...
CW010P-008 ......G.H......K..........K...
CW010A-022 ........SF.....S.M....A...D...
CW010A-028 ........SF.....S.M....A...D...
CW010H-561 ........SF.....S.M....A...D...
CW010H-565 ........SF.....S.M....A...D...
CW010G-001 ........SF.....S.M....A...D...
CW010G-002 ........SF.....S.M....A...D...
CW010F-002 ........SF.....S.M....A...D...
CW010F-003 ........SF.....S.M....A...D...
CW010F-004 ........SF.....S.M....A...D...

CW012D-752 NNNTRKSIHM--GPGRAFFATGEIIGDIRQ
CW012D-751 S.......NI.........YT.........K
CW012D-753 ..............................
CW012D-776 S.......NI.........YT.........K
CW012P-002 ........PI.........YT.........K
CW012P-003 ..............................
CW012A-031 ..............................
CW012A-036 ..................YT..........
CW012A-039 ..............................
CW012H-531 ..............................
CW012H-533 ........PI.........YT.........K
CW012H-536 ................Y.............
CW012H-539 ................Y.............
CW012G-002 ................Y.............
CW012G-003 ..............................
CW012F-004 ................Y.............
CW012F-003 ................Y.............
CW012B-001 ..............................
CW012B-002 ..............................

CW037D-364 NNNTRKSIHI--GPGRAFYTTGGIIGNIRQ
CW037D-366 ..................A..E.......
CW037D-361 ......................E.......
CW037P-374 ......................D.......
CW037A-013 ..................A..D.......
CW037A-014 ..............................
CW037A-015 D.................A..D.......

CW048D-225 SNNTRKSIPI--GPGRAFYTTGEIIGDIRQ
CW048D-222 N............G............K
CW048D-223 ..............................
CW048P-078 ........H.....................
CW048P-081 ........H.....................
CW048P-082 ........H.....................
CW048P-079 ........H.....................
CW048A-241 ............................K
CW048A-247 ............................K
CW048A-244 ............................K

CW002D-153 HNNTRKSIHI--GPGRAFYATGEIIGDIRQ
CW002D-152 ..............................
CW002P-129 N.............................
CW002P-131 ......................Q.T.....
CW002P-130 ......................Q.T.....
CW002P-128 ......................Q.T.....
CW002P-086 ......................Q.T.....
CW002A-251 N.............................
CW002A-254 N.............................
CW002A-259 N.............................
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b) V2 domain

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           1         11        21        31
           |         |         |         |
    NL43_1 ISTSIRDKVQKEYAFFYKLDIVPID--------NTSYRLI

CW010D-010 ITTRMRDRVQREYALFYKLDVVPLD-------NDTSYRLI
CW010D-007 ..........................................
CW010D-009 ..........................................
CW010D-0A6 ..........................................
CW010D-005 ...S.K.KM.T...F........I.NNDNTSYGNN.....
CW010P-006 ..........................................
CW010P-007 ..........................................
CW010P-008 ..........................................
CW010A-022 ...S.K.KM.T...F........I.NNDNTSYSNN.....
CW010A-028 ...S.K.KM.T...F........I.NNDNTSYGNS.....
CW010H-561 TA.S.K.KM.T...F........I.DNDNTSYGNN.....
CW010H-565 ...S.K.KM.T...F........I.NNDNTSYGNN.....
CW010G-001 ...S.K.KM.T...F........I.NNDNTSYGNN.....
CW010G-002 ...S.K.KM.T...F........I.NNDNTSYGNN.....
CW010F-002 T..S.K.KM.T...F........I.DNDNTSYGNN.....
CW010F-003 T..S.K.KM.T...F........I.DNDNTSYGNN.....
CW010F-004 T..S.K.KM.T...F........I.DNDNTSYGNN.....

CW012D-752 ITTSMKDKVQKTYALFYKLDVVPIN-GD--NTT--SYRLI
CW012D-751 ........................E.............
CW012D-753 ..........................................
CW012D-776 ........................E.............
CW012P-002 ......................ME.-.D.-K-NT.....
CW012P-003 ......................ME.-.D.-K-NT.....
CW012A-031 ......................M..E....K.SS.....
CW012A-036 ......................MEPR.DN.DSNT.....
CW012A-039 ......................MEPR.DN.DSKT.....
CW012H-531 ......................MEPR.DN.DSKT.....
CW012H-533 ......................ME.-.D.-K-NT.....
CW012H-536 ......................M..E....K.SS.....
CW012H-539 ......................M..E....K.SS.....
CW012G-002 ......................MEVPMDD.K.SSN...V
CW012G-003 ......................M..E....K.SS.....
CW012F-004 ...N................I.M-.E.DN..........
CW012F-003 ...N................I.M-.E.DN..........
CW012B-001 ......................MEPR.DN.DSNT.....
CW012B-002 ......................E.-.DNK.I.S.....

CW037D-364 ITTNIRDKIQREYAIFYKIDIVPI----DNKTA--RYRLI
CW037D-366 .A..............M.L.......N.D--NAS....
CW037D-361 ........T....................E.........
CW037P-374 ..................L.........D.V...H...
CW037A-013 ................L.L.S.......N.T.......
CW037A-014 ............L...L.L.........D.I...H...
CW037A-015 ................L.L.S.......N.T.......

CW048D-225 ITASIRDKMQREYALFYKLDVVPIDRD--NTS----YRLI
CW048D-222 ............S...........................
CW048D-223 ..........................................
CW048P-078 ..T.....................K..D...........
CW048P-081 ..T.....................K..D...........
CW048P-082 ..T.....................K..D...........
CW048P-079 ..T.....................--.............
CW048A-241 ..GG...................I.ND...........
CW048A-247 ..GG...................I.ND...........
CW048A-244 ..GG...................I.ND...........

CW002D-153 IT-NIGNKIKEEYALFHELDVVQIDST--------SYTLK
CW002D-152 ..........................................
CW002P-129 ..TGR...RQK.....YK....P..KNNTNKG.NAN...I
CW002P-131 ..TSR...RQK.....YK....PM.KNNTNKG.NAN...I
CW002P-130 ..TGR...RQK.....YK....P..KNNTKKG.NAN...I
CW002P-128 ..TSR...RQK.....YK....PM.KNNTNKG.NAN...I
CW002P-086 ..TGR...RQK.....YK....P..KNNTNKG.NAN...I
CW002A-251 -.TS...E.................KNN.NRS...-....
CW002A-254 -.TS...E.................KNN.NRS...-....
CW002A-259 -.TS...E.................KNN.NRS...-....
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Fig. 3. Alignment of the V3 (a) and V2 (b) domains of the envelope glycoproteins. Clones are labelled according to the key provided in Fig. 2. The domains are defined as described (Douglas *et al.*, 1997). The alignment and pretty-print were produced within GDE using HIV-1_{NL43} (subtype B) as a reference sequence. In the alignment, (—) shows gaps introduced to improve the alignment, (.) shows where there is agreement with the base-line sequence selected for each patient's virus.

PBMC isolation of HIV-1 from the other three patients (CW037, CW048 and CW002) showed individual selection patterns compared with sequences derived from *in vivo* samples but again all clones were predicted to be of NSI phenotype based on their V3 sequences (Fig. 3a). Since the selection profiles for virus isolation from the five patient samples were different, it was difficult to predict which of the substitutions might be important in HIV-1 isolation by cell co-culture. It is well established that physico-chemical properties are potentially useful in predicting which regions of aligned protein subfamilies are important in determining function (Caffrey *et al.*, 2000). The physico-chemical properties (hydrophobicity, volume and charge) were calculated by domain (defined in Douglas *et al.*, 1997) for each patient's groups of *in vivo*- and isolate-derived glycoprotein clones. Student's *t*-test was applied to monitor the significance of alterations observed. The significant difference profiles varied dramatically between patients, with CW037 showing the least significant differences (CW037 < CW012 < CW048 < CW002 < CW010; results not shown). However, when considering *in vivo*- and isolate-derived physico-chemical properties across the five patient samples, only the V2 domain showed significant and consistent trends in terms of increases in negative charge in virus isolates (Fig. 3b).

The *env* gene contains coding information for three other genes. The *vpu* gene sequence overlaps the region encoding the signal peptide of the HIV-1 glycoprotein and the second exons of *tat* and *rev* genes are contained within the gp41 cytoplasmic tail coding region. The translated sequences for these three proteins did not show any remarkable features compared with sequences reported in the Los Alamos Database but did show the individuality of HIV-1 strains infecting each patient. All *vpu* reading frames were open and terminated at the usual position. The majority of *tat* exons terminated after encoding 29 amino acids but four clones from CW012 (D-751, D-776, P-002, H-533) were truncated prematurely, whilst another (A-039) showed an amino acid extension due to mutation of the normal stop codon. Similarly, the majority of *rev* exons terminated after encoding 91 amino acids except for eight clones from CW012, which contained a three amino acid extension (ECC) due to mutation of the normal stop codon. Two further clones, CW048P-082 and CW037D-364, contained premature terminations. It has been shown that fully functional and attenuated *rev* genes can persist in HIV-1-infected, asymptomatic individuals (Iversen *et al.*, 1995). Generally there was high similarity between sequences derived directly from patient samples (DNA/plasma) and those of their respective HIV-1 isolates.

Discussion

We have addressed the virus selection issue during isolation of HIV-1 in PBMCs and a variety of HVS T cell lines. For the two patients studied, isolation was achieved in all co-culture combinations except for CW010/AC. Although a range of virus replication rates were observed, similar peak p24 titres were obtained, except for CW010/CN-1 co-culture (Fig. 1). Other *in vitro* studies have shown that whilst the majority of donor cells are susceptible to infection with a range of HIV-1 strains, they support replication of particular HIV-1 strains to widely varying extents (Chang *et al.*, 1994; Spira & Ho, 1995). Variation in replication rates may be related to the donor PBMCs carrying different HLA haplotypes. Maximal heterozygosity at HLA class I loci has been associated with slow progression to AIDS and AIDS-related death (Carrington *et al.*, 1999). In the present study, we are likely to have removed major effects relating to HLA class I loci by CD8-depletion of all PBMC preparations. However, those relating to HLA class II loci may remain and CN-1/2 contain two loci (DRB1*0401 and DRB4*0101), which have been associated with a significantly decreased risk of AIDS development (Kroner *et al.*, 1995). In addition to HLA differences, the lower replication rates observed in HVS T cells compared with PBMCs may be related to alterations in the cell population introduced during the immortalization process since CP-1 and the PBMC pool are of the same origin, as is the case for CN-1 and CN-2. Further studies of the HVS T cell lines, in terms of cell-surface markers, will shed light on the latter issue. Of the HVS T cell lines used, CN-2 supported the highest HIV-1 replication rates. Unlike conventional T cell lines, 80–95% of CN-2 cells expressed CD4, CD45RO, CD26 and CD7, and both CCR5 and CXCR4 co-receptors (Vella *et al.*, 1999a). Cells expressing CD26 and CD7 have been reported to be a subset of cell types that are particularly susceptible to HIV-1 infection *in vivo* (Callebaut *et al.*, 1998; Wallace *et al.*, 2000).

Virus isolates from CW010 and CW012 could be passaged in PBMCs only and all displayed an NSI phenotype. Furthermore, for CN-2/PBMC isolate pairs, the virus neutralization profiles were similar (Table 2). This suggested similar selection following isolation in PBMCs and HVS T cell lines. *Env* gene sequencing showed there to be selection following isolation for sequences different to those present in the *in vivo* (provirus and virus) situations. Whilst these selections involved different amino acid positions and regions of the glycoproteins for each patient's isolates, the selections were similar in PBMCs and HVS T cell lines. Similarly, for patients where PBMC isolation only was performed, individual selection patterns were observed. In comparing the isolation patterns for all five patients, only the V2 domain showed a significant and consistent trend in terms of increases in negative charge compared with the *in vivo* situations. This observation supports the NSI phenotype of the CW010 and CW012 isolates, as it has been shown that SI isolates carry more positive charge in V2 compared with NSI isolates and small changes in V2 can affect virus tropism (Cornelissen *et al.*, 1995; Koito *et al.*, 1995; Vella *et al.*, 1999c). That this domain has important structure–function determination properties is shown by the conservation of Asp 21 (Fig. 3b), which is important for virus

replication, and the observation that length differences in V2 modulate exposure of the CD4 binding site and the immunoreactivity of gp120 (Wang *et al.*, 1995; Fox *et al.*, 1997). These properties probably account for V2 being targeted by HIV-neutralizing antibodies (Shotton *et al.*, 1995; Wu *et al.*, 1995).

The translation products of the three accessory gene reading frames (*vpu*, *tat* and *rev*) were studied for evidence of selection following virus isolation. Vpu has two biological functions, down-regulation of CD4 and enhancement of virus release from infected cells (Miller & Sarver, 1997). Tat and Rev are *trans*-acting nuclear regulatory proteins, which play essential roles in the HIV-1 replication cycle (Pollard & Malim, 1998; Jeang *et al.*, 1999). All Vpu carboxy-termini were intact and whilst there were patient/virus strain-specific sequences, there was neither evidence for selection following virus isolation nor the presence of inactivating substitutions. Tat and Rev exon 2 were patient/virus strain-specific and only for CW010 was there evidence of selection following virus isolation. The majority of exons were intact and probably functional, as there were no remarkable amino acid substitutions compared with sequences presented in the database. Overall, the majority of expression-competent *env* genes rescued in the course of this study should be functional in terms of the overlapping *vpu*, *tat* and *rev* genes.

The results presented here show that there are no significant differences in biological phenotype between HIV-1 isolates rescued in HVS T cell lines and PBMCs. Therefore, given their more stable phenotype, HVS T cells are an equivalent/better host for rescuing primary HIV-1 isolates than PBMCs. From *env* gene sequencing studies, it was observed that both cell types selected for viruses carrying more negative charge in the V2 domains of their glycoproteins, a property that corresponds with the observed NSI phenotype of all isolates. The significance of this selection is unknown but it may represent a common selection for viral sequences that display an M-tropic/NSI phenotype, as has been observed in different individuals during seroconversion to HIV-1 (Zhu *et al.*, 1993). Consequently, the selection seen following HIV-1 isolation in PBMCs/HVS T cells may reflect that occurring during natural infection.

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