Table 3. Comparison of amino acid length, number of potential *N*-linked glycosylation sites, V3 sequences and co-receptor usage between anti-retroviral drug-selected and control-passaged KP-1 variants

	Passage no.	Genetic diversity*	Mean ENV_{1-474} length (range)†	Mean V1/V2 length (range)	Mean V3 length (range)	Mean V4 length (range)	Mean PNGs (range)	V3 region		Geno2 pheno (%)§
								Prevalence (%)	Sequence‡	
Baseline	0	0.056	472 (461–480)	69 (60–74)	34 (33–34)	30 (29–31)	24 (22–28)	41.9	CTRPNNNTRKGIHIGPGKFYATGAIIGDIRQAHC	41.2
								22.6	.....V.....	41.2
								16.1	....-..I.....T.R..T.RD...N..K...	1.7
								13.0	....-..I.....T.R..T.KT...N.KK...	2.9
								3.2	....-..I.....	7.4
								3.2	.....D.....	55.3
Passage control	8	0.0070	463 (462–463)	62	34	29	23 (22–23)	100.0	.....V.....	41.2
RAL-selected virus	8	0.0070	480	74	34	31	28 (26–29)	100.0	.....	41.2
3TC-selected virus	6	0.020	478 (475–480)	74	34	31 (29–31)	27 (25–28)	83.3	.....	41.2
SQV-selected virus	11	0.0040	474	71	34	31	26	100.0	.....	41.2
MVC-selected virus	7	0.0080	469 (468–469)	69	33	29	24 (23–24)	100.0	....-..I....R..T.R..T.KT...N.KK...	1.7

*Overall mean distance.

†Sequence from gp120 SP to the V5 region (aa 1–474).

‡V3 sequences of each variant are shown. Dots denote sequence identity and dashes indicate a deletion mutation.

§Prediction of viral co-receptor tropism using Geno2pheno based on a selectable 'false positive rate'.

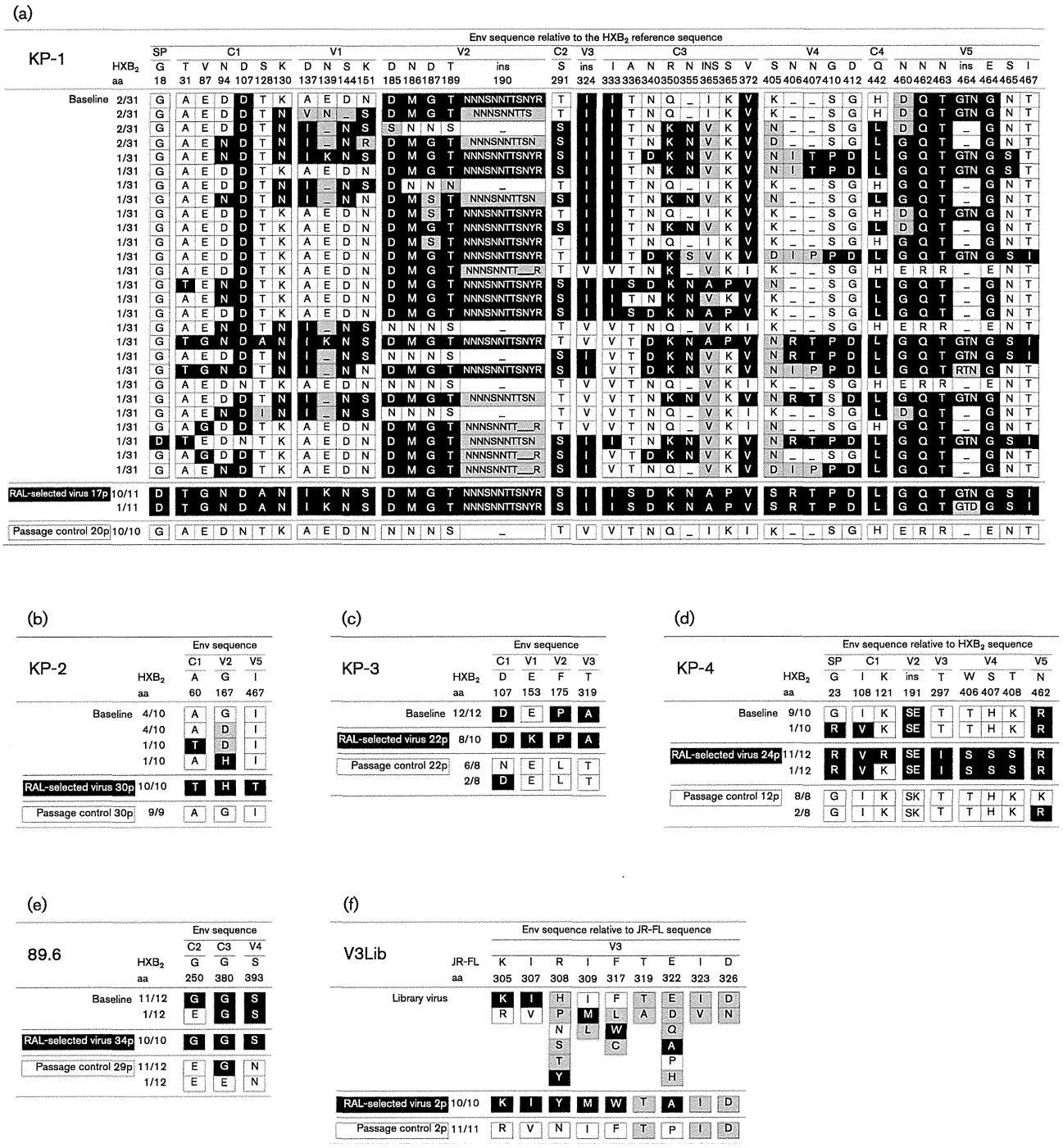


Fig. 1. *Comparison of the gp120 sequences between RAL-selected and control-passaged viruses.* The gp120 sequences of baseline, RAL-selected and the passage-control viruses were aligned for KP-1 (a), KP-2 (b), KP-3 (c), KP-4 (d) and strain 89.6 (e). Each amino acid in (a)–(e) is numbered relative to the HIV-1 HXB_2 reference sequence. The V3 sequences from the JR-FL-V3Lib baseline library, RAL-selected and passage-control viruses were aligned (f). Filled cells denote the most dominant amino acids observed in RAL-selected variants at the latest passage, open cells denote the most dominant amino acids observed in the passage-control variants at the latest passage and shaded cells show amino acids deleted by the end of both passages, whilst '–' indicates a deletion mutation. The number of passages is indicated, e.g. 17p for passage 17.

In vitro selection of KP-1 variants by 3TC, SQV and MVC

To determine whether other HIV drugs also changed the route of adaptation to the target cells, we attempted to select KP-1 variants using a reverse transcriptase inhibitor (3TC), a protease inhibitor (SQV) and a CCR5 inhibitor (MVC). As shown in Fig. 2(f), the pattern of clustering at distinct positions between the selected isolates and the passage-control variants was similar to that observed for the RAL-selected variants. The selected variants showed decreased diversity in the gp120 sequences; however, the length of the gp120, V1/V2 and V4 sequences increased (apart from in the MVC-selected variants). In addition, the number of PNGs within gp120 was higher than that in the control (Table 3). We also compared the V3 sequences between the passage-control and each of the drug-selected variants. The V3 sequences in all the SQV-selected variants and 83.3 % of those in the 3TC-selected variants, were comparable with those in the RAL-selected variants. This was not the case for the passage controls. Comparison of variants passaged with RAL and 3TC showed that the length of the V1/V2 and V4 regions and the number of PNGs was similar; however, these parameters were different in the SQV-selected variants (Table 3). This indicated that the time at which a drug acts (e.g. during the early or late phase of the HIV life cycle) influences the selection of Env sequences. During selection with MVC, CXCR4-tropic variants were selected from the baseline mixture after seven passages.

Taken together, these results suggested that, in treated cells, different classes of anti-HIV drugs may suppress the variability of quasi-species during *in vitro* selection via a route different from that in untreated cells.

DISCUSSION

This study evaluated the impact of anti-HIV drugs on the Env bottleneck in bulk HIV-1 primary isolates during selection *in vitro*. RAL-, 3TC- and SQV-selected variants of the unique viral isolate, KP-1, harbouring both X4 and R5 variants and with a very high level of baseline viral diversity, were used to study the final destination (genetic bottleneck) of a large variety of Env sequences. Interestingly, the phylogenetic clustering of RAL-selected KP-1 variants was completely different from that of non-drug-treated controls (Fig. 2). Our results also confirmed differences in the length of the gp120, V1/V2 and V4-loop regions and in the number of PNGs (Tables 2 and 3).

It is not clear why viruses cultured under pressure from the non-Env-directed drug RAL result in different *env* genotypes compared with those without the drug. Thus, we cloned the *IN–env* region of the proviral genome from passaged viruses and sequenced the *env* and *IN* regions on the same cloned plasmid, and compared them among the baseline and passages 1, 2, 8 and 17 of the KP-1 virus. Under low concentrations of the IN inhibitor RAL, K7 was selected for at a late passage after accumulation of the other three amino acids, K111, D278 and H216, in IN. During the sequential accumulation of these four amino acids (K111, D278, H216 and K7), the RAL-selected Env sequences at passage 17 (the Env sequences shown as filled boxes in Fig. 1) sequentially accumulated mutations in the same proviral genome (Fig. S1, available in JGV Online). However, we did not find a clone including both the RAL-selected Env at passage 17 and RAL-selected IN at passage 17 in the baseline or each passaged virus, except for in the last passage. We also examined the gp120 and IN sequences of the 3TC- and SQV-selected KP-1 variants. Compared with the RAL-selected region, the variable regions of gp120 in these selected variants were very similar to each other, except for the V1/V2 region (Fig. S2). However, the passage-control variant was very different from the drug-selected variants (Fig. 1a). Furthermore, the IN sequences were different in each passaged virus: K111/D278/H216/K7 in RAL-selected, R111/D278/Q216/R7 in 3TC-selected, K111/D278/H216/R7 in SQV-selected and R111/N278/Q216/R7 in virus without drug treatment (underlined residues indicate amino acids different from those in viruses without drug treatment). To explain these results, we believe that, under pressure from anti-HIV drugs (non-entry ARVs), the virus might show a primitive reaction to select for the Env sequence and recombine from quasi-species to gain advantage for entry and/or enhance replication in target cells. Meanwhile, IN was selected from quasi-species by a direct and/or indirect effect of RAL-induced pressure. The combination of both selective pressures may affect the selection for Env and IN during adaptation in drug-treated conditions (Figs 1a and S2). These results suggest that non-entry inhibitors, such as RAL, 3TC and SQV, might also affect cell adaptation to PM1/CCR5 cells.

Many *in vivo* studies have reported the effects of the anti-HIV drug-induced bottleneck on the *env* gene (Charpentier *et al.*, 2006; Delwart *et al.*, 1998; Ibáñez *et al.*, 2000; Kitrinos *et al.*, 2005; Nijhuis *et al.*, 1998; Nora *et al.*, 2007; Sheehy *et al.*, 1996; Zhang *et al.*, 1994). However, these studies had several limitations. Because viruses were placed under *in vivo* selective pressure using at least two anti-HIV drugs and by the host immune response, it is difficult to separate the different effects and to draw clear conclusions, particularly *in vivo*. Delwart *et al.* (1998) and Kitrinos *et al.* (2005) avoided some of these limitations by employing a heteroduplex tracking assay, although *in vivo* peculiarities still remained. Therefore, we used an *in vitro* selection system using unique bulk primary isolates established in our laboratory (Hatada *et al.*, 2010; Shibata *et al.*, 2007; Yoshimura *et al.*, 2006, 2010b) to observe the effects of the anti-retroviral drug-induced bottleneck on the *IN* and *env* genes.

This selection provides a sensitive approach for analysing virus population dynamics. The effectiveness of ARV drugs can be examined during the *in vitro* passage of a single variant or mixture of variants without being affected by many of the factors encountered *in vivo*. In addition,

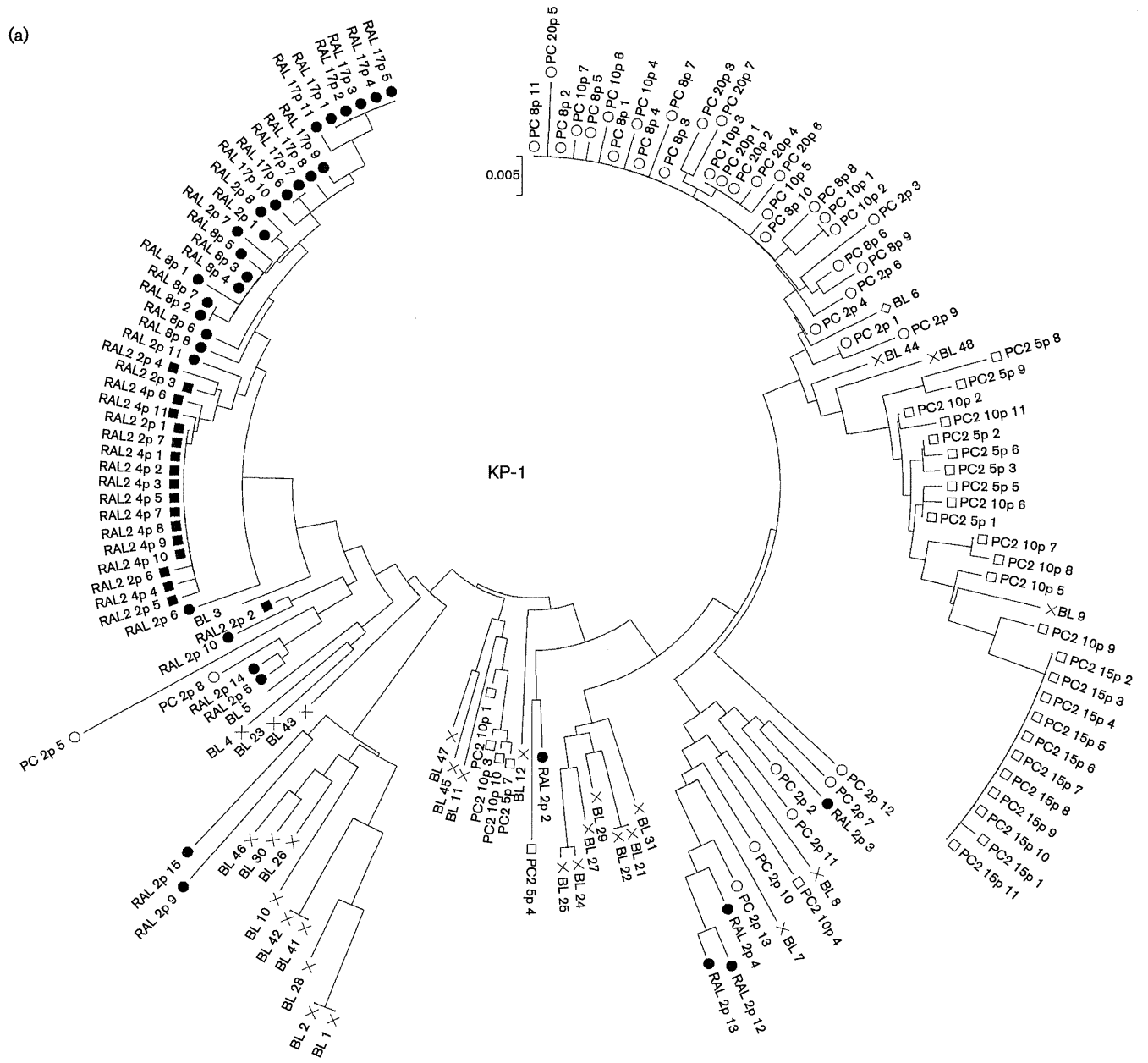
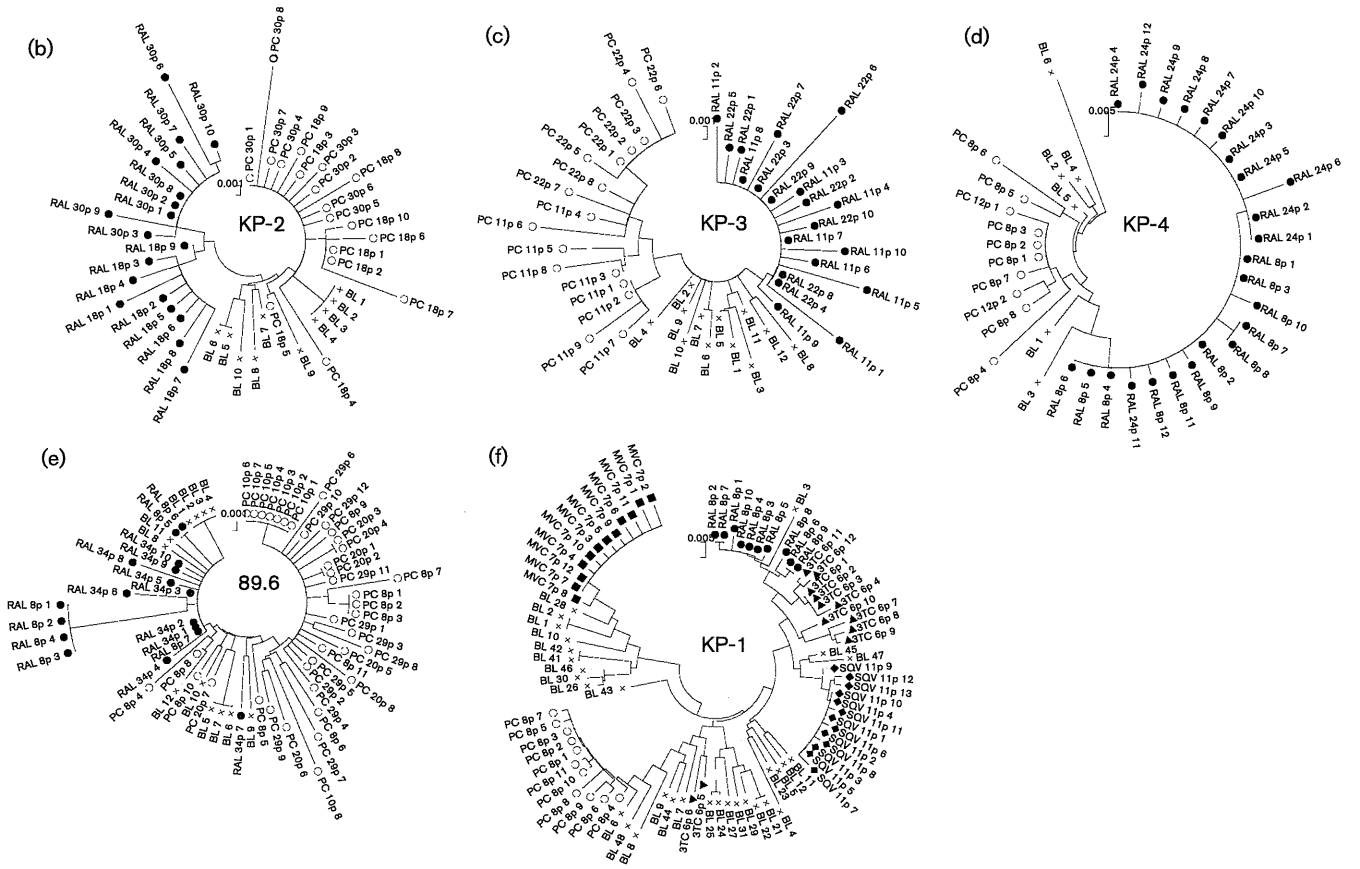


Fig. 2. Phylogenetic analyses of the Env regions from *in vitro*-passaged viruses selected with or without ARV drugs. (a–e) Phylogenetic trees were constructed using gp120 SP–V5 sequences from RAL-selected and passage-control variants of KP-1 (a), KP-2 (b), KP-3 (c), KP-4 (d) and strain 89.6 (e). An '×' represents baseline (BL) variants, and closed and open symbols represent RAL-selected (RAL) and passage-control (PC) variants, respectively. In (a), the results of the second experiment are indicated as RAL2 and PC2, respectively. (f) A phylogenetic tree was constructed using gp120 SP–V5 sequences from RAL-, 3TC-, SQV-, MVC-selected and control-passaged variants of KP-1. ○, Control variants after eight passages; ●, RAL-selected variants after eight passages; ▲, 3TC-selected variants after six passages; ◆, SQV-selected variants after 11 passages; ■, MVC-selected variants after seven passages. The trees were constructed using the neighbour-joining algorithm embedded within the MEGA software.

differences in the Env sequences between the baseline and selected variants can be compared after any number of passages. The results of the present study provide important information that will enhance our understanding of the drug-induced genetic bottleneck. This phenomenon can be examined *in vitro* using bulk primary isolates treated with or without drugs.

Recently, several new ARV drugs have been licensed for use in HIV-1-infected patients. MVC, approved in 2006, is the



first CCR5 inhibitor (Gulick *et al.*, 2008). One important advantage associated with this drug is the absence of cross-resistance with previously available ARV compounds (Gulick *et al.*, 2008; Steigbigel *et al.*, 2008). However, as is usual with anti-HIV drugs, resistant variants with mutations in the Env, gp120 and gp41 sequences are induced both *in vivo* and *in vitro* (Anastassopoulou *et al.*, 2009; Berro *et al.*, 2009; Tilton *et al.*, 2010; Yoshimura *et al.*, 2009, 2010a). As shown in the present study, distinct Env sequences from each quasispecies might be selected by the different anti-HIV drugs (e.g. length of the V1/2 and/or V4 regions, V3 region depletion and the number of PNGs). Moreover, many of the novel anti-retroviral drugs in pre-clinical trials are viral entry inhibitors (e.g. PRO140, ibalizumab, BMS-663068 and PF-232798; Jacobson *et al.*, 2010; McNicholas *et al.*, 2010; Nettles *et al.*, 2011; Stupple *et al.*, 2011; Toma *et al.*, 2011). Therefore, it is necessary to examine whether such entry inhibitors are effective when used alongside conventional drugs.

In conclusion, we studied the genetic bottleneck in bulk primary HIV-1 isolates from untreated patients and drugs targeting the Env (and other) regions. The results showed, for the first time, the presence of drug-selected Env sequences in these isolates. Although our observations were based on a limited number of HIV-1 isolates and need to be confirmed by independent studies, we believe that they provide a new paradigm for HIV-1 evolution in the new combination ARV therapy era.

METHODS

Patients and isolates. Primary HIV-1 isolates were isolated from four drug-naïve patients in our laboratory (KP-1–4) and passaged in phytohaemagglutinin-activated PBMCs. Infected PBMCs were then co-cultured for 5 days with PM1/CCR5 cells (a kind gift from Dr Y. Maeda; Maeda *et al.*, 2008; Yusa *et al.*, 2005) and the culture supernatants were stored at −150 °C (Hatada *et al.*, 2010; Shibata *et al.*, 2007; Yoshimura *et al.*, 2006, 2010b).

After isolation of the primary viruses, we checked the sensitivity of each primary isolate to MVC. The KP-1 isolate was relatively MVC-resistant compared with KP-2 and KP-3 (54 vs 5.9 and 8.7 nM, respectively). KP-1 became MVC sensitive after eight passages in PM1/CCR5 cells [IC_{50}, 3.4 nM; Geno2pheno value (see below), 41.2 %], whilst under the pressure of MVC, KP-1 became highly resistant to MVC after eight passages (IC_{50}, >1000 nM; Geno2pheno value, 1.7 %). These results indicated that the bulk KP-1 isolate used in this study harboured primarily R5 viruses with X4- or dual-tropic viruses as a minor population.

Cells, culture conditions and reagents. PM1/CCR5 cells were maintained in RPMI 1640 (Sigma) supplemented with 10 % heat-inactivated FCS (HyClone Laboratories), 50 U penicillin ml^{-1}, 50 μg streptomycin ml^{-1} and 0.1 mg G418 (Nacalai Tesque) ml^{-1}. MVC, RAL and SQV were kindly provided by Pfizer, Merck & Co. and Roche Products, respectively. 3TC was purchased from Wako Pure Chemical Industries.

The laboratory-adapted HIV-1 strain 89.6, which was obtained through the NIH AIDS Research and Reference Reagent Program, was propagated in phytohaemagglutinin-activated PBMCs. The viral-competent library pJR-FL-V3Lib, which contains 176 bp V3–loop DNA fragments with 0–10 random combinations of amino acid substitutions, was introduced into pJR-FL, as described previously (Yusa *et al.*, 2005).

***In vitro* selection of HIV-1 variants using anti-HIV drugs.** The four primary HIV isolates (KP-1–4), strain 89.6 and JR-FL-V3Lib were treated with various concentrations of RAL and used to infect PM1/CCR5 cells to induce the production of RAL-selected HIV-1 variants, as described previously, with minor modifications (Hatada *et al.*, 2010; Shibata *et al.*, 2007; Yoshimura *et al.*, 2006, 2010b). Briefly, PM1/CCR5 cells (4×10^4 cells) were exposed to 500 $TCID_{50}$ HIV-1 isolates and cultured in the presence of RAL. Virus replication in PM1/CCR5 cells was monitored by observing the cytopathic effects. The culture supernatant was harvested on day 7 and used to infect fresh PM1/CCR5 cells for the next round of culture in the presence of increasing concentrations of RAL. When the virus began to propagate in the presence of the drug, the compound concentration was increased further. Proviral DNA was extracted from lysates of infected cells at different passages using a QIAamp DNA Blood Mini kit (Qiagen). The proviral DNAs obtained were then subjected to nucleotide sequencing. *In vitro* selection of the KP-1 isolate using SQV, 3TC and MVC was also performed using the procedure described above.

Amplification of proviral DNA and nucleotide sequencing. Proviral DNA was subjected to PCR amplification using PrimeSTAR GXL DNA polymerase and Ex-*Taq* polymerase (Takara), as described previously (Hatada *et al.*, 2010; Shibata *et al.*, 2007; Yoshimura *et al.*, 2006, 2010b). The primers used were 1B and H for the gp120 region (Hatada *et al.*, 2010; Shibata *et al.*, 2007; Yoshimura *et al.*, 2006, 2010b), IN 1F (5′-CAGACTCACAATATGCATTAGG-3′) and IN 1R (5′-CCTGTATGCAGACCCCAATATG-3′) for the *IN* region, and IN 1F and H for the *IN*–gp120 region. The first-round PCR products were used directly in a second round of PCR using primers 2B and F (Hatada *et al.*, 2010; Shibata *et al.*, 2007; Yoshimura *et al.*, 2006, 2010b) for gp120, IN 2F (5′-CTGGCATGGGTACCAGCACACAA-3′) and IN 2R (3′-CCTAGTGGGATGTGTACTTCTGAACTTA-3′) for *IN*, and IN 2F and F for *IN*–gp120. The PCR conditions used were as described above. The second-round PCR products were purified and cloned into a pGEM-T Easy Vector (Promega) or pCR-XL-TOPO Vector (Invitrogen), and the *env* and *IN* regions in both the passaged and selected viruses were sequenced using an Applied Biosystems 3500xL Genetic Analyzer and a BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems). Phylogenetic reconstructions were generated using the neighbour-joining method embedded in the MEGA software (http://www.megasoftware.net) (Tamura *et al.*, 2007). Overall, mean distances for viral diversity were also calculated using MEGA software. The number and location of putative PNGs were estimated using *N*-GlycoSite (http://www.hiv.lanl.gov/content/sequence/GLYCOSITE/glycosite.html) from the Los Alamos National Laboratory database.

Susceptibility assay. The sensitivity of the passaged viruses to various drugs was determined as described previously with minor modifications (Hatada *et al.*, 2010; Shibata *et al.*, 2007; Yoshimura *et al.*, 2006, 2010b). Briefly, PM1/CCR5 cells (2×10^3 cells per well) in 96-well round-bottomed plates were exposed to 100 $TCID_{50}$ of the viruses in the presence of various concentrations of drugs and incubated at 37 °C for 7 days. The IC_{50} values were then determined using a Cell Counting Kit-8 assay (Dojindo Laboratories). All assays were performed in duplicate or triplicate.

Predicting co-receptor usage by the V3 sequence. HIV-1 tropism was inferred using Geno2pheno [coreceptor] program, with a false rate positive (FPR) value of 5.0 %, which is freely available (http://coreceptor.bioinf.mpi-inf.mpg.de/index.php). This genotyping tool more accurately predicts virological responses to the CCR5 antagonist MVC in ARV-naïve patients than a reference phenotypic tropism test (Sing *et al.*, 2007).

Statistical analyses. Pairwise comparisons of the different parameters between variants in the two groups was calculated using the homoscedastic *t*-test. A P value of <0.05 was considered statistically significant.

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Increased infectivity in human cells and resistance to antibody-mediated neutralization by truncation of the SIV gp41 cytoplasmic tail

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The role of antibodies in protecting the host from human immunodeficiency virus type 1 (HIV-1) infection is of considerable interest, particularly because the RV144 trial results suggest that antibodies contribute to protection. Although infection of non-human primates with simian immunodeficiency virus (SIV) is commonly used as an animal model of HIV-1 infection, the viral epitopes that elicit potent and broad neutralizing antibodies to SIV have not been identified. We isolated a monoclonal antibody (MAb) B404 that potently and broadly neutralizes various SIV strains. B404 targets a conformational epitope comprising the V3 and V4 loops of Env that intensely exposed when Env binds CD4. B404-resistant variants were obtained by passaging viruses in the presence of increasing concentration of B404 in PM1/CCR5 cells. Genetic analysis revealed that the Q733stop mutation, which truncates the cytoplasmic tail of gp41, was the first major substitution in Env during passage. The maximal inhibition by B404 and other MAbs were significantly decreased against a recombinant virus with a gp41 truncation compared with the parental SIVmac316. This indicates that the gp41 truncation was associated with resistance to antibody-mediated neutralization. The infectivities of the recombinant virus with the gp41 truncation were 7,900-, 1,000-, and 140-fold higher than those of SIVmac316 in PM1, PM1/CCR5, and TZM-bl cells, respectively. Immunoblotting analysis revealed that the gp41 truncation enhanced the incorporation of Env into virions. The effect of the gp41 truncation on infectivity was not obvious in the HSC-F macaque cell line, although the resistance of viruses harboring the gp41 truncation to neutralization was maintained. These results suggest that viruses with a truncated gp41 cytoplasmic tail were selected by increased infectivity in human cells and by acquiring resistance to neutralizing antibody.

Keywords: SIV, gp41, truncation, infectivity, resistance, neutralization, antibody

INTRODUCTION

The RV144 trial demonstrated 31% vaccine efficacy for preventing human immunodeficiency virus type 1 (HIV-1) infection (Rerks-Ngarm et al., 2009). Antibodies against the HIV-1, particularly against the V1/V2 loops, correlate inversely with infection risk (Haynes et al., 2012). Further recent isolation of monoclonal antibodies (MAbs) that neutralize a broad range of HIV-1 strains suggest the possibility for developing a vaccine that can induce cross-neutralizing antibodies effective for various HIV-1 strains (Kwong and Mascola, 2012). Although non-human primate models of simian immunodeficiency virus (SIV) infection can facilitate the evaluation of immunogens, epitopes and immune correlates, no potent and broad neutralizing MAb against SIV had been available.

To understand the mechanisms involved in neutralization of infectivity by antibodies in an SIV model, we recently isolated MAb B404 from a SIVsmH635FC-infected rhesus macaque, which potently and broadly neutralizes various SIV strains, such as SIVsmE543-3, SIVsmE660 and the neutralization-resistant variants, genetically diverse SIVmac316, and highly neutralization-resistant SIVmac239 (Kuwata et al., 2011). The B404 epitope, which comprises the V3 and V4 loops of Env and is intensely exposed by ligation of Env to CD4, is the target for potent and broad neutralization of SIV (Kuwata et al., 2013). Vigorous induction of B404-like neutralizing antibodies using the specific VH3 gene with a long complementarity-determining region 3 loop and λ light chain was observed in four SIVsmH635FC-infected macaques. The B404-resistant variants were induced by passaging viruses in the presence of increasing concentrations of B404. Genetical analysis of the gp120 region of B404-resistant variants revealed that the mutations in the C2 region of Env were important for the resistance to antibody-mediated neutralization (Kuwata et al., 2013).

In the present study, we further analyzed B404-resistant variants and determined the precise region responsible for the resistance to antibody-mediated neutralization. Genetic analysis of viruses during passage in the presence of B404 as well as phenotypic analysis using recombinant viruses revealed that a truncation of the gp41 cytoplasmic tail was the primary step leading to escape from neutralization.

MATERIALS AND METHODS

CELLS

PM1 (Lusso et al., 1995), PM1/CCR5 (Yusa et al., 2005), and HSC-F (Akari et al., 1999) cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% fetal bovine serum (FBS). TZM-bl (Platt et al., 1998; Derdeyn et al., 2000; Wei et al., 2002; Takeuchi et al., 2008) and 293T (DuBridge et al., 1987) cells were maintained in Dulbecco's modified Eagle's medium containing 10% FBS.

GENETIC ANALYSIS OF B404-RESISTANT VARIANTS

The induction of variants resistant to Fab-B404 (Kuwata et al., 2011) from SIVmac316 (Mori et al., 1992) harboring full-length gp41 was performed as described previously (Yoshimura et al., 2006; Hatada et al., 2010; Kuwata et al., 2013). Briefly, 5,000 $TCID_{50}$ (50% tissue culture infectious dose) SIVmac316 was incubated with 5 ng/ml Fab-B404 for 30 min at 37°C. Then, 5×10^4 PM1/CCR5 cells were added to the virus–Fab mixture. After incubation for 5 h, cells were washed with phosphate-buffered saline (PBS) and resuspended in RPMI 1640 supplemented with 10% FBS without Fab-B404. The culture supernatant was harvested 7 days later and used to infect fresh PM1/CCR5 cells for the next round of culture in the presence of increasing concentrations of Fab-B404. Proviral DNA samples were extracted from cells using a QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany) after 8, 17, 20, 23, and 26 passages as well as from P26C cells obtained after 26 passages in the absence of Fab-B404. The gp120 region was amplified using Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA, USA) with primers SEnv-F (5′-ATG GGA TGT CTT GGG AAT CAG C-3′) and SER1 (5′-CCA AGA ACC CTA GCA CAA AGA CCC-3′). The whole *env* gene was amplified with primers SRev-F (5′-GGT TTG GGA ATA TGC TAT GAG-3′) and SEnv-R (5′-CCT ACT AAG TCA TCA TCT T-3′). The polymerase chain reaction (PCR) products were cloned using a TA cloning kit (Invitrogen), and subjected to sequencing. Nucleotide sequences were aligned and analyzed phylogenetically using Molecular Evolutionary Genetics Analysis version 5 (MEGA5) (Tamura et al., 2011).

CONSTRUCTION OF INFECTIOUS MOLECULAR CLONES WITH THE Env REGION FROM B404-RESISTANT VARIANTS

One of the clones from passage 26, P26B404 clone 26, was selected for construction of recombinant viruses, because this clone had mutations typical of the major population of P26B404 variants. Infectious molecular clones SS, SN, and NS were generated by replacing fragments *SphI–SacI* [nucleotides (nt) 6,446–9,226], *SphI–NheI* (nt 6,446–8,742), and *NheI–SacI* (nt 8,742–9,226) with the corresponding regions of SIVmac316, respectively. Mutants F277V and N295S, which have point mutations at amino acid residues 277 and 295 of Env, respectively, were constructed by PCR mutagenesis using the SIVmac316 plasmid as template. The changes from phenylalanine (TTC) to valine (GTC) in F277V and asparagine (AAT) to serine (AGT) in N295S were introduced using primers F277VFw (5′-TTG GTT TGG CGT CAA TGG TAC TAG GGC-3′), F277VRv (5′-GTA CCA TTG ACG CCA AAC CAA G-3′), N295SFw (5′-GGC AAT AGT AGT AGA ACC ATA ATT AG-3′), and N295SRv (5′-AAT TAT GGT TCT ACT ACT ATT GCC-3′). Mutant and parental SIVmac316 plasmids were transfected into 293T cells using X-tremeGENE 9 DNA Transfection Reagent (Roche Molecular Biochemicals, Mannheim, Germany). After 2 days, the supernatants containing viruses were filtered (0.45 μm) and stored at −80°C.

ANALYSIS OF VIRAL INFECTIVITY

For determination of $TCID_{50}$ in PM1 and PM1/CCR5 cells, 5×10^4 cells in 50 μl were inoculated with 50 μl serially diluted virus stocks in a 96-well plate and cultured for 2 weeks. Virus replication was judged by observation of cytopathic effects (CPE) by light microscopy. The $TCID_{50}$ in TZM-bl cells was determined by measuring luciferase activities. Briefly, 100 μl medium, 50 μl serially diluted virus stock, and 50 μl 1×10^4 cells containing 37.5 μg/ml diethylaminoethyl (DEAE) dextran were added to the wells of a 96-well plate. The plate was then incubated at 37°C for 2 days. After washing with PBS, cells were lysed with 30 μl cell lysing buffer (Promega, Madison, WI, USA) for 15 min at room temperature (RT) and then 10 μl of cell lysate was transferred to a 96-well white solid plate (Coster, Cambridge, MA, USA). Luciferase activity was measured using a Centro XS3 LB960 microplate luminometer (Berthold Technologies, Bad Wildbad, Germany) and a luciferase assay system (Promega). The $TCID_{50}$ was calculated according to the formula of Reed and Muench (1938).

Infectivity of viruses in PM1, PM1/CCR5, and HSC-F cells was evaluated by detecting infected cells using flow cytometry as described previously (Kuwata et al., 2011). Briefly, PM1 and PM1/CCR5 cells were adjusted to 1×10^6 cells/ml and HSC-F cells were adjusted to 5×10^6 cells/ml. Aliquots of 100 μl cells per well in a 24-well plate were inoculated with 100 μl of diluted virus stocks. After incubation for 6 h, 800 μl fresh medium was added to wells. One-half of the cells in each well were collected at 4, 7, and 10 days post-inoculation. Cells were washed with PBS and fixed with IC Fixation Buffer (eBioscience, San Diego, CA, USA). After washing with Permeabilization Buffer (eBioscience) twice, the cells were intracellularly stained with 4 μg/ml (50 μl) anti-p27 Fab, B450 (Kuwata et al., 2011) by incubation for 20 min at RT. The cells were then incubated with 50 μl anti-HA antibody (1:200; 3F10, Roche Molecular Biochemicals) for 20 min at RT followed by incubation with 50 μl of anti-rat-FITC (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 20 min at RT. The stained cells were analyzed using a FACSCalibur (BD Biosciences, Franklin Lakes, NJ, USA). Frequencies of infected cells were determined by comparison with an uninfected control. Data analysis was performed using FlowJo (TreeStar, San Carlos, CA, USA).

All infectivity experiments were performed at least twice and the representative results are shown.

ANALYSIS OF NEUTRALIZING ACTIVITIES

The Fab clones B404 and K8, isolated from an SIV-infected macaque (Kuwata et al., 2011), and murine MAb M318T (Matsumi et al., 1995) were used to examine the sensitivity of viruses to antibody-mediated neutralization in TZM-bl cells as described previously (Kuwata et al., 2011). Briefly, 100 μl serially diluted antibodies in duplicate were incubated with 200 $TCID_{50}$ (50 μl) of virus in a 96-well plate. After incubation for 1 h at 37°C, 100 μl

of 1×10^5 TZM-bl cells/ml containing 37.5 μg/ml DEAE dextran were added. After incubation for 2 days, luciferase activities were measured as described above for the analysis of viral infectivity. The 50% inhibitory concentrations (IC_{50}) and maximal percent of inhibition (MPI) were calculated from the average values by non-linear regression using Prism5 (GraphPad Software, San Diego, CA, USA).

Sensitivity to neutralization by B404 in macaque cells was analyzed using HSC-F cells, a cynomolgus macaque cell line immortalized by infection with *Herpesvirus saimiri* (Akari et al., 1999). Fab-B404 was serially diluted and 50 μl aliquots were mixed with 50 μl undiluted or 10-fold diluted virus in a 96-well plate. After 1 h incubation at 37°C, 2×10^5 cells in 100 μl were added to each well and cultured for 1 day. The infected cells were washed twice with PBS, resuspended in 200 μl fresh medium, and cultured in a new 96-well plate. Viral infection was examined 4 days post-inoculation by intracellular staining of p27, as described above for the analysis of viral infectivity. Infectivity was determined in duplicate and the average value was used for the analysis of neutralization.

All neutralizing assays were performed at least twice and the representative results are shown.

WESTERN BLOTTING ANALYSIS OF VIRAL PROTEINS

Cells and supernatants were collected from six-well plate 2 days after transfection of 293T cells with infectious molecular clones, as previously described (Yuste et al., 2005). Supernatants were filtered (0.45 μm) and clarified by centrifugation for 10 min at 3,000 rpm. The clarified supernatants were centrifuged at 13,200 rpm for 90 min at 4°C, and the viral pellets were resuspended in 1 ml PBS and centrifuged again. Pellets were then dissolved in 80 μl sample buffer [62.5 mM Tris–HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 25% glycerol, 5% 2-mercaptoethanol, 0.01% bromophenol blue]. Cells were washed with PBS and lysed in 300 μl sample buffer. Samples of virions and cell lysates were boiled for 5 min, and the proteins were separated by SDS-polyacrylamide gel electrophoresis using SuperSep Ace 5–20% (Wako Pure Chemical Industries, Osaka, Japan). Proteins were transferred to an Immun-Blot PVDF Membrane (Bio-Rad Laboratories, Hercules, CA, USA). The membrane was blocked with 5% skim milk TBS-T (Tris-buffered saline containing 0.1% Tween 20) for 1 h at RT, and then washed three times with TBS-T. For the detection of gp120, the membrane was incubated overnight at 4°C with 1 μg/ml M318T (Matsumi et al., 1995) in 5% skim milk TBS-T. After washing three times with TBS-T, the membrane was incubated with anti-mouse immunoglobulin G (IgG) peroxidase (1:4,000, Santa Cruz Biotechnology) for 1 h at RT. The membrane was washed three times with TBS-T and once with TBS, and then TMB solution (KPL, Gaithersburg, MD, USA) was added to develop color. Viral proteins gp41 and p26 were similarly examined using crude supernatants from bacterial culture producing B408 and B450 (Kuwata et al., 2011), which were mixed with the same amount of 5% skim milk TBS-T. The membrane was incubated with anti-HA-HRP antibody (1:1,000; Roche Molecular Biochemicals) and Chemi-Lumi One L (Nacalai Tesque, Kyoto, Japan), and viral proteins were visualized using ImageQuant LAS 4000 (GE Healthcare, Piscataway, NJ, USA)

RESULTS

EVOLUTION OF VIRUSES DURING PASSAGE UNDER THE PRESSURE OF Fab-B404

To select for variants resistant to MAb B404, an antibody that targets a conformational epitope comprising the gp120 V3 and V4 loops, we passaged SIVmac316 that possesses a full-length gp41 in PM1/CCR5 cells in the presence of increasing concentrations of Fab-B404. The virus recovered at passage 26 (P26B404) was resistant to neutralization by B404 (V3/V4) and other antibodies, MAbs K8 (CD4i) and M318T (V2), that target epitopes other than that recognized by B404 (Kuwata et al., 2013). The region covering the whole *env* gene were amplified by PCR and cloned from viruses at passage 8, 17, 20, 23, and 26. The nucleotide sequences were phylogenetically analyzed to show the evolution of B404-resistant variants (**Figure 1**). The first major mutation was a change from glutamine (CAG) to a stop codon (TAG) at 733rd amino acid residue of Env. The Q733stop substitution in the gp41 cytoplasmic domain was observed in 12 of 14 clones at passage 8 and in all clones thereafter. Another stop codon (W782stop) was the second

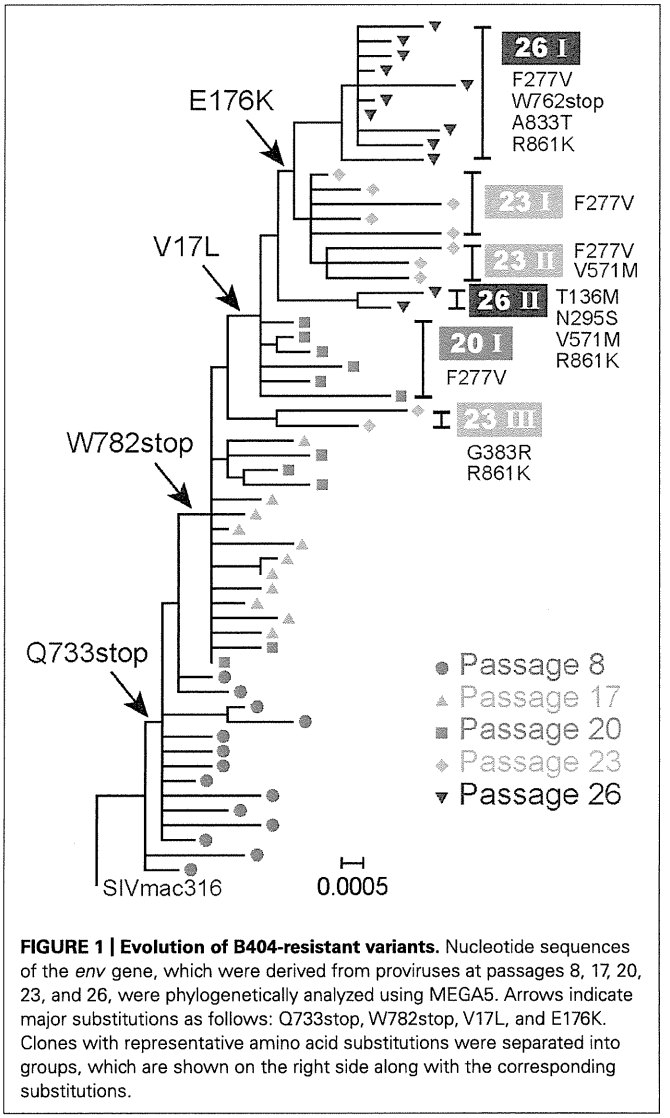


FIGURE 1 | Evolution of B404-resistant variants. Nucleotide sequences of the *env* gene, which were derived from proviruses at passages 8, 17, 20, 23, and 26, were phylogenetically analyzed using MEGA5. Arrows indicate major substitutions as follows: Q733stop, W782stop, V17L, and E176K. Clones with representative amino acid substitutions were separated into groups, which are shown on the right side along with the corresponding substitutions.

major mutation, which was detected after 17 passages. Substitutions V17L in the signal peptide and E176K in the V2 loop emerged after 20 and 23 passages, respectively, although the E176K substitution was also observed in P26C, control viruses after 26 passages in the absence of B404 (**Table 1**). In addition to these substitutions, most of clones acquired the F277V substitution in the late stage of evolution, except for one group at passage 26 which has the N295S substitution (see **Figure 1**, group 26II). Group 26II was clearly distinguished from group 26I by amino acid substitutions, such as T136M, N295S, and D571M/E (**Table 1**), suggesting two lineages of variants in P26B404.

These results demonstrated that the first step in acquiring resistance to B404 was the truncation of gp41. Although substitutions in gp120, represented by F277V, might contribute to the resistance to a high concentration of B404, 20 passages were required for the emergence of these substitutions.

Table 1 | Frequency* of amino acid substitutions in Env clones from B404-resistant variants after 26 passages.

Substitution	Region	P26B404		P26C
		I	II	
	gp120	**(*n* = 22)**	**(*n* = 8)**	**(*n* = 14)**
V17L	Signal peptide	**100%**	**100%**	0.0%
G62S	C1	0.0%	0.0%	21.4%
M67V/L/T	C1	4.5%	0.0%	21.4%
A68T	C1	0.0%	0.0%	**92.9%**
T136M	V1	4.5%	**87.5%**	0.0%
T137I	V1	0.0%	0.0%	14.3%
K141E/R	V1	0.0%	12.5%	7.1%
E176K	V2	**90.9%**	12.5%	35.7%
F277V	C2	**100%**	0.0%	0.0%
N295S	C2	0.0%	**100%**	0.0%
Q341H	V3	13.6%	12.5%	14.3%
D374N	C3	0.0%	0.0%	28.6%
K403R	V4	0.0%	12.5%	7.1%
W441R	C4	4.5%	0.0%	7.1%
	gp41	**(*n* = 10)**	**(*n* = 2)**	**(*n* = 7)**
F528S/L	Extracellular	20.0%	0.0%	0.0%
D571M/E	Extracellular	10.0%	**100%**	0.0%
Q733stop	Cytoplasmic	**100%**	**100%**	0.0%
W762stop	Cytoplasmic	**100%**	0.0%	0.0%
W782stop	Cytoplasmic	**100%**	0.0%	0.0%
A833T	Cytoplasmic	**90.0%**	0.0%	0.0%
R839K	Cytoplasmic	0.0%	0.0%	**57.1%**
R861K	Cytoplasmic	**100%**	**100%**	0.0%

Percentages of substitutions in populations P26B404 and P26C, which were obtained after 26 passages in the presence and absence of B404, respectively, are shown. The P26B404 population is separated into two subpopulations according to the phylogenetic analysis in **Figure 1. All the substitutions that are observed in more than one clone are shown here. Boldface indicates substitutions dominant (>50%) in each population. The numbers of clones analyzed for the gp120 and gp41 regions are shown in parentheses.*

TRUNCATION OF gp41 CONFERRED RESISTANCE TO ANTIBODY-MEDIATED NEUTRALIZATION

To analyze effect of substitutions in B404-resistant variants on resistance to neutralization, recombinant viruses were constructed (**Figure 2**). The *env* region of SIVmac316 was replaced by that of P26B404 clone 26, which had substitutions typical to the P26I group. The resultant molecular clones SS, SN, and NS had substitutions in the entire *env* region, gp120 and gp41 from P26B404I, respectively. SS and NS were predicted to have a truncated gp41 with no other mutation in gp41, because the Q733stop substitution was the first substitution in gp41. Point mutants with substitutions F277V and N295S, which were representative mutations at late passages, were also constructed by PCR mutagenesis.

These mutant viruses were examined for their sensitivity to neutralization by three MAbs B404 (V3/V4 conformational), K8 (CD4i), and M318T (V2). The neutralization of SS that contain the entire *env* region from P26B404I was similar to those of P26B404, indicating that the *env* region is responsible for the

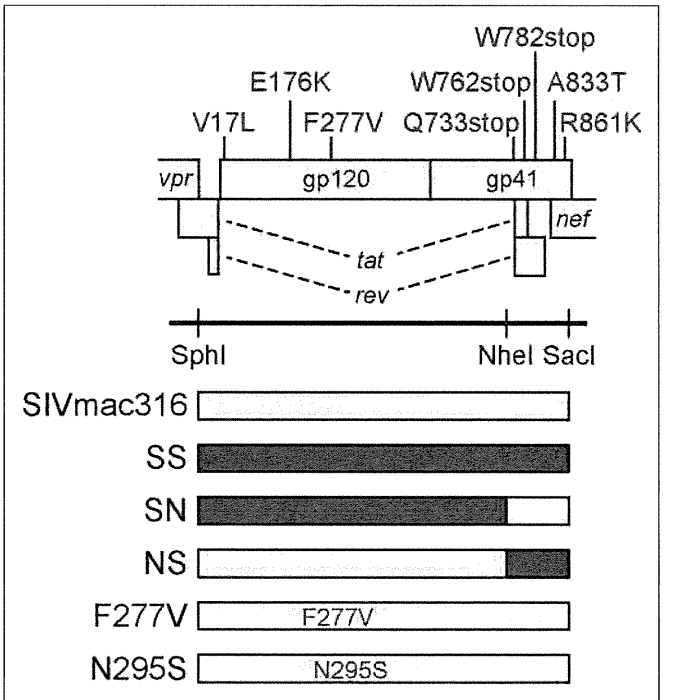
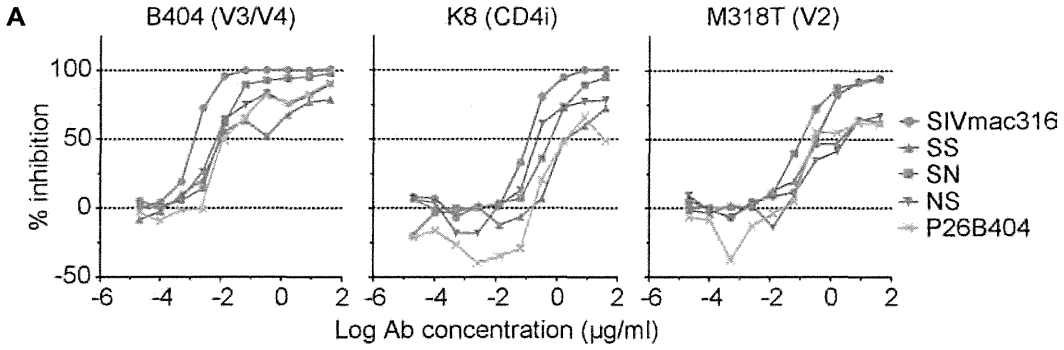


FIGURE 2 | Construction of infectious SIV clones with B404-resistant mutations. The open reading frames of the SIV genome are shown along with the Env substitutions in the B404-resistant variant typical to P26B404I. Full-length SIV clones were constructed from parental SIVmac316 by replacing the *env* region with that of the B404-resistant variant (blue) using *Sph*I, *Nhe*I, and *Sac*I sites. The resultant virus SS contains all of the Env substitutions present in P26B404I. Viruses SN and NS contain substitutions in gp120 and gp41, respectively. Point mutants F277V and N295S were constructed by inserting the substitutions F277V and N295S into SIVmac316, respectively.

resistance to neutralization (**Figure 3A**). Recombinants SN and NS, which have substitutions in gp120 and gp41 from P26B404I, respectively, showed varying degrees of resistance. The IC_{50} values of SN and NS against B404 were intermediates between the parental SIVmac316 and the neutralization-resistant P26B404. Maximal inhibition reached a plateau at 73.8, 82.3, and 81.9% in SS, NS, and P26B404, respectively, but the MPI value of SN (94.9%) was close to that of SIVmac316 (100%; **Figure 3B**). Neutralization resistance to anti-CD4i MAb K8 was characterized by decreases in the IC_{50} value of SN and the MPI of NS. Neutralization by anti-V2 MAb M318T was even enhanced in SN, although NS showed the resistance comparable to those of SS and P26B404. The decreases in MPI values were commonly observed for the neutralization of NS by the three MAbs (**Figure 3B**). Resistance to neutralization was not significantly detected by the point mutants F277V and N295S, except for the neutralization of F277V by K8 (4.3-fold decrease of IC_{50} value). These results indicated that the entire *env* region, including substitutions in both gp120 and gp41, was responsible for the full-resistance of P26B404 to neutralization. The decrease of MPI values for NS suggested that truncation of gp41 by the Q733stop substitution, the first major substitution in viral evolution, was important to escape from the neutralizing antibodies.

INCREASED INFECTIVITY FOR HUMAN CELLS BY SIV WITH A TRUNCATED gp41

Truncation of gp41 in SIV is associated with the adaptation to human cells (Hirsch et al., 1989; Kodama et al., 1989), which may partially contribute to neutralization resistance (Yuste et al., 2005). To explore the mechanism of neutralization resistance of P26B404, the infectivity of recombinant viruses was analyzed by determining the $TCID_{50}$ values of virus stocks prepared by transfection of 293T cells (**Table 2**). The $TCID_{50}$ values in all the human cells tested were significantly higher for SS and NS viruses with truncated gp41 than



B

Neutralization profile of mutant viruses

Virus	B404 (V3/V4)		K8 (CD4i)		M318T (V2)	
	IC_{50}	MPI	IC_{50}	MPI	IC_{50}	MPI
SIVmac316	1.3E-03 (1.0)	100 (0.0)	1.1E-01 (1.0)	99.0 (0.0)	3.3E-01 (1.0)	94.3 (0.0)
SS	1.9E-02 (14.5)	73.8 (-26.2)	1.8E+00 (16.3)	67.9 (-31.1)	9.4E-01 (2.8)	65.5 (-28.8)
SN	9.1E-03 (6.8)	94.9 (-5.1)	5.9E-01 (5.3)	94.7 (-4.4)	1.0E-01 (0.3)	92.5 (-1.8)
NS	6.8E-03 (5.1)	82.3 (-17.7)	2.1E-01 (1.9)	77.0 (-22.0)	1.7E+00 (5.2)	67.6 (-26.7)
F277V	3.8E-03 (2.8)	100 (0.0)	4.8E-01 (4.3)	99.6 (0.5)	3.0E-01 (0.9)	94.8 (0.5)
N295S	2.3E-03 (1.7)	99.6 (-0.4)	1.4E-01 (1.2)	99.9 (0.8)	3.1E-01 (0.9)	97.0 (2.7)
P26B404	1.7E-02 (12.5)	81.9 (-18.1)	8.2E-01 (7.3)	54.6 (-44.5)	2.5E-01 (0.8)	60.0 (-34.3)

Fold change of IC_{50}
> 10
3-10
< 3

% change of MPI
< -20
-10- to -20
> -10

FIGURE 3 | Sensitivities of viruses with B404-resistant mutations to neutralization by MAbs. (A) Sensitivities of mutant viruses SS, SN, and NS to neutralization by MAbs Fab-B404 (anti-V3/V4), Fab-K8 (anti-CD4i), and M318T (anti-V2) are shown. Neutralization of parental SIVmac316 and B404-resistant P26B404 is also shown as controls sensitive and resistant to neutralization, respectively. **(B)** The sensitivities to neutralization are represented by values for IC_{50} (μg/ml) and MPI (%). Viruses were examined for their sensitivities to neutralization by MAbs Fab-B404, Fab-K8, and M318T in TZM-bl cells. The IC_{50} and MPI values were determined using Prism 5. The fold-change of IC_{50} was calculated by dividing the IC_{50} value by that of the parental SIVmac316. Percent change of MPI was calculated by subtracting the MPI value from that of SIVmac316. These changes are shown in parentheses and significant changes are indicated by magenta (IC_{50}: >10; MPI: < −20) and orange (IC_{50}: 3–10; MPI: −20 to −10) highlighting.

parental SIVmac316 and SN, in which gp41 is intact. In particular, NS showed a striking increase in $TCID_{50}$ values, which were 7,100-, 1,000-, and 140-fold higher than those of parental SIVmac316 in PM1, PM1/CCR5, and TZM-bl cells, respectively. These results indicate that truncation of gp41 caused by the Q733stop substitution increases viral infectivity for human cells.

To compare viral infectivity in human and macaque cells, viral infection was monitored after inoculation of PM1 and PM1/CCR5 human cells and the HSC-F cynomolgus macaque cell line with varying dilutions of virus stocks (**Figure 4**). Consistent with the $TCID_{50}$ analysis, a higher frequency of infected cells was detected earlier in PM1 and PM1/CCR5 cells inoculated with NS than the parental SIVmac316. In contrast, SN showed decreased infectivity in PM1 and PM1/CCR5 cells, apparently because PM1 cells were not infected by a 1,000-fold diluted SN stock. Although the $TCID_{50}$ values of SS were much higher than those of SIVmac316, the replication kinetics of SS were similar to those of SIVmac316 in PM1 and PM1/CCR5 cells. These results suggest that gp41 truncation increases infectivity for human cells and that the substitutions in gp120 of P26B404I are associated with slow and poor replication compared with that of SIVmac316.

Infectivity for macaque cells was more significantly affected than that for human cells by the substitutions in gp120 of P26B404I (**Figure 4**, lower panels). Infected cells were detected in HSC-F cells inoculated with 1,000-fold diluted virus stocks of SIVmac316 and NS, but viral infection in HSC-F cells was limited to a low frequency even by inoculation with 10-fold diluted virus stocks of SS and SN. Truncation of gp41 did not significantly affect replication in HSC-F macaque cells, although truncation of gp41 was disadvantageous for replication in primary T cell cultures from macaques (Hirsch et al., 1989; Kodama et al., 1989).

These results demonstrate that gp41 truncation strikingly increases infectivity for human cells, but not for macaque cells, and that the substitutions in gp120 decrease infectivity in human and macaque cells. Truncation of gp41, which conferred extremely high infectivity for PM1/CCR5 cells, may be the first step to escape from neutralization and the substitutions in gp120 may be the second step to replicate in the presence of high concentration of B404.

Table 2 | Infectivity* of viruses with substitutions from P26B404.

Viruses	PM1	PM1/CCR5	TZM-bl
SIVmac316	4.2E+02 (1.0)	1.4E+03 (1.0)	9.6E+04 (1.0)
SS	2.9E+05 (710)	4.7E+05 (350)	6.3E+06 (66)
SN	2.0E+03 (4.8)	8.4E+03 (6.2)	2.9E+05 (3.1)
NS	2.9E+06 (7,100)	1.4E+06 (1,000)	1.4E+07 (140)

**Infectivity is shown by the $TCID_{50}$/ml values of the viruses, which were prepared by transfection of 293T cells, in PM1, PM1/CCR5, and TZM-bl cells. The fold-change, which was calculated by dividing the mutant $TCID_{50}$/ml value by that of the parental SIVmac316, is shown in the parentheses.*

INCREASED INCORPORATION OF Env INTO VIRIONS IN SIV WITH TRUNCATED gp41

Incorporation of Env into virions was examined using these recombinant viruses, because increased infectivity by gp41 truncation was suggested to be associated with the Env content of virions (Manrique et al., 2001; Zhu et al., 2003, 2006; Yuste et al., 2004, 2005). Analysis of viral proteins in cells and supernatants from transfected 293T cells revealed that incorporation of Env into virions was significantly high in SS and NS viruses with the Q733stop substitution (**Figure 5**). MAb to gp120 showed a higher amount

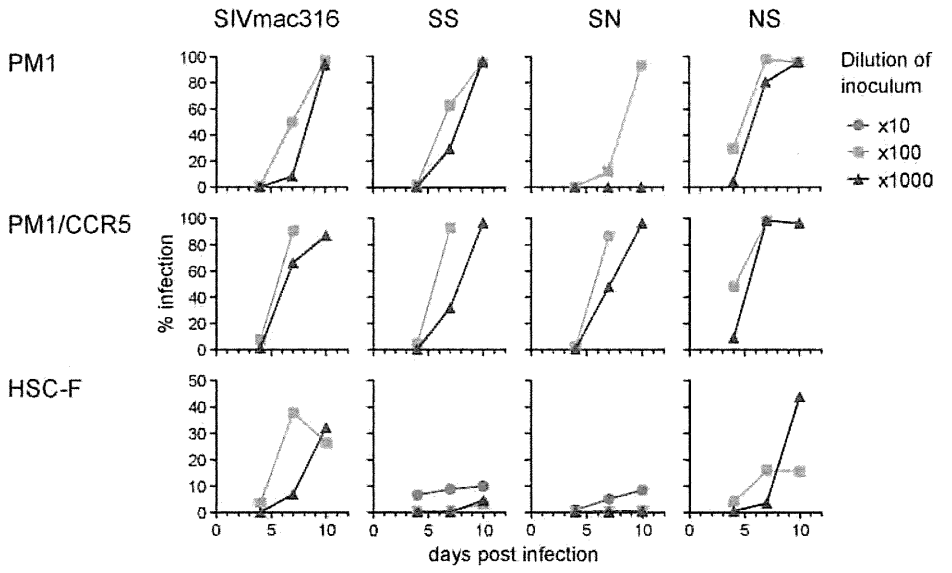


FIGURE 4 | Replication of B404-resistant viruses in human and macaque cells. Frequencies of infected cells at 4, 7, and 10 days post-infection are shown. PM1 and PM1/CCR5 human T cells and HSC-F cynomolgus macaque T cells were infected with the various dilutions of SIVmac316, SS, SN, and NS. Infected cells were detected using flow cytometry after intracellular staining by B450 (anti-p27 Fab).

of gp120 and gp160 in virions from SS and NS than those from SN and the parental SIVmac316, although the production of Env proteins in the transfected cells was at the same level among all the viruses (**Figure 5A**). MAb to gp41 also demonstrated that truncated gp41 was more abundant in virions compared with full-length gp41 (**Figure 5B**). Semi-quantification by densitometric scanning of gp41 and p26 images suggested that the levels of gp41 amount per virion in SS and NS were 12- and 44-fold higher than that of SIVmac316, respectively, after adjusting virion numbers using the p26 amounts. In contrast to the increased amount of Env proteins in virions from viruses with truncated gp41, the level of Gag p27 in virions was low in SS and NS compared with those in SN and SIVmac316 (**Figure 5C**). This indicates that the Env content per virion, which was normalized by the amount of p27, was significantly high in viruses with truncated gp41. These results suggest that truncation of gp41 by the Q733stop substitution enhances incorporation of Env into virions.

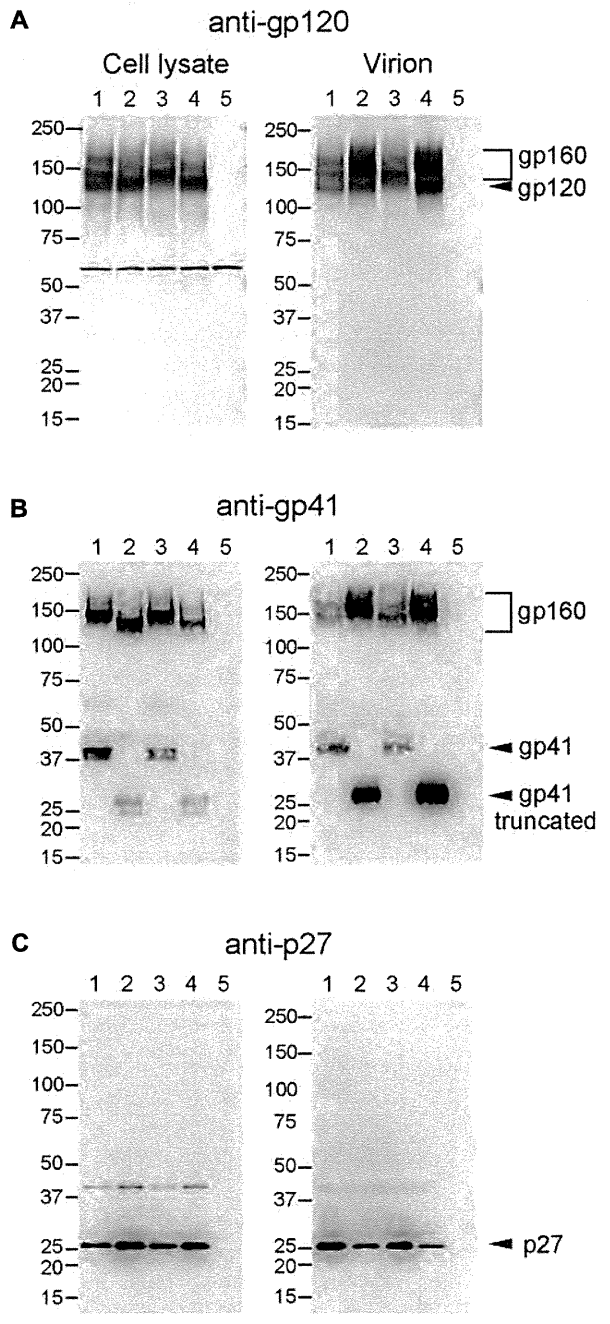


FIGURE 5 | Effect of B404-resistant mutations on the incorporation of Env into virions. Cell lysates (left panels) and virions (right panels) of SIVmac316 (lane 1), SS (lane 2), SN (lane 3), NS (lane 4), and mock-infected control (lane 5), which were prepared from transfected 293T cells, were analyzed by Western blotting. Viral proteins gp120 **(A)**, gp41 **(B)**, and p27 **(C)** were detected using MAbs M318T (anti-gp120 V2), B408 (anti-gp41 cluster I), and B450 (anti-p27), respectively.

NEUTRALIZATION RESISTANCE OF SIV WITH TRUNCATED gp41 IN MACAQUE CELLS

The analysis of infectivity of recombinant viruses suggested that the resistance to neutralization by truncation of gp41 might be due to adaptation to human cells. To examine this hypothesis, sensitivity to neutralization by B404 was determined in HSC-F macaque cells using SIVmac316 and NS, which showed similar infectivity for HSC-F cells (**Figure 4**). In flow cytometric analysis, infection in the presence or absence of B404 demonstrated that the high sensitivity of SIVmac316 and resistance of NS to neutralization were maintained in HSC-F cells (**Figure 6**). The frequency of infected cells decreased from 41.5% to the background level (2.03%) in inoculation with the undiluted stock of SIVmac316. In contrast, infection with NS, even with a 10-fold diluted virus stock, was significant in HSC-F cells in the presence of B404 (**Figure 6A**). Neutralization of NS in HSC-F cells was characterized by a decrease in maximal inhibition (**Figure 6B**), which was also observed in TZM-bl cells (**Figure 3A**). The magnitude of resistance of NS to B404 was greater when infection was performed using the undiluted stock compared with the 10-fold diluted stock, raising the possibility that B404 did not inhibit infection with a high titer of viruses. However, the resistance of NS was shown by infection with a low titer of NS, in which the frequency of infected cells in the absence of B404 (23.8%) was lower than infection with undiluted SIVmac316 (41.5%). Further, immunoblotting analysis revealed that the amount of virions was higher in the virus stock of SIVmac316 than that of NS (**Figure 5**).

These results indicate that gp41 truncation by the Q733stop substitution contributes to neutralization resistance of viruses in macaque cells. This suggests that the resistance to neutralization by truncation of gp41 is not due to the adaptation to human cells. The Q733stop substitution, the first major mutation during passages in the presence of B404, might be selected because it facilitates adaptation of virus to human cells and imparts resistance to antibody.

DISCUSSION

In the present study, truncation of the cytoplasmic tail of gp41, which was caused by the Q733stop substitution in Env, was the first major mutation detected during passage of SIV in the presence of the neutralizing antibody B404. Analysis of recombinant viruses suggested that the gp41 truncation was selected by their resistance to neutralizing antibody, which was characterized by the decrease of maximal inhibition compared with viruses with intact gp41, and

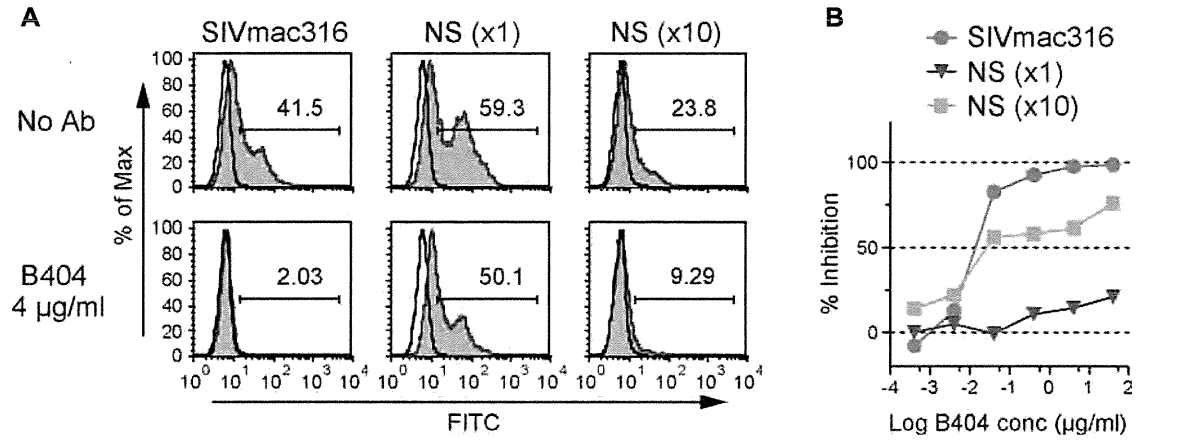


FIGURE 6 | Contribution of gp41 truncation to neutralization resistance in infection of macaque cells. (A) Infection of HSC-F cells in the presence (4 μg/ml) or absence of Fab-B404 is shown. HSC-F cells were inoculated with undiluted SIVmac316 and NS, and 10-fold diluted NS. Viral infection was monitored 4 days post-infection using flow cytometry after intracellular staining by B450 (anti-p27 Fab). **(B)** Neutralization of SIVmac316 and NS by B404 in HSC-F cells are shown. Percent inhibition was calculated by comparison with the infected culture in the absence of B404.

increased infectivity for human cells. The premature stop codon in the gp41 cytoplasmic region was frequently detected in SIV strains propagated in human cell culture *in vitro*, such as the original SIVmac316 clone, SIVmac1A11 and 17E-Fr (Hirsch et al., 1989; Kodama et al., 1989; Mori et al., 1992; Bonavia et al., 2005; Vzorov et al., 2005). The truncation of gp41 is considered as an adaptation of SIV to replication in human cell culture, because the premature stop codon rapidly reverted to express full-length gp41 after infection of rhesus primary cell culture *in vitro* and rhesus macaques *in vivo* (Hirsch et al., 1989; Kodama et al., 1989). Mutant viruses harboring the gp41 truncation showed increased infectivity for human cells, although the effects on infectivity varied depending on the SIV strain and the length of the gp41 truncation (Manrique et al., 2001; Yuste et al., 2004, 2005; Vzorov et al., 2005, 2007). The enhancement effect of gp41 truncation on incorporation of Env into virions, which were demonstrated by quantification of viral proteins in virions (Yuste et al., 2004) and electron tomography analysis of Env trimers on virions (Zhu et al., 2003, 2006), was partly associated with the increased infectivity caused by gp41 truncation (Manrique et al., 2001; Yuste et al., 2004, 2005). Because expression of Env on the cell surface is regulated by the cytoplasmic domain of gp41, truncation of gp41 may increase Env density on both cells and virions (LaBranche et al., 1995; Berlioz-Torrent et al., 1999; Postler and Desrosiers, 2013). Consistent with these studies, infectivity for human cells and Env incorporation into virions was enhanced by gp41 truncation in the present study. Although the mechanism responsible for increasing viral infectivity caused by gp41 truncation remains unclear, the high virion Env content may contribute to the efficient replication of viruses with truncated gp41 in human cells.

The effect of gp41 truncation on susceptibility to antibody-mediated neutralization is controversial, perhaps due to the SIV strains used for the analyses. Because most of prototypic SIV clones with truncated gp41 were macrophage-tropic, CD4-independent, and neutralization-sensitive (Mori et al., 1992; Bonavia et al., 2005; Vzorov et al., 2005), the truncation of gp41 was assumed responsible for the high sensitivity to neutralization. However, the resistance to neutralization by gp41 truncation was shown using the E767stop mutant of SIVmac316 (Yuste et al., 2005). This is consistent with our results using SIVmac316 harboring the Q733stop substitution, indicating that gp41 truncation contributes to resistance of SIVmac316 to neutralization. The increased infectivity of viruses with gp41 truncation in human cells may partially play a role in resistance by overcoming antibody-mediated neutralization via efficient attachment and entry of viruses to cells. However, we showed that gp41 truncation was also associated with neutralization resistance in macaque cells, in which gp41 truncation did not significantly affect infectivity. This suggests that the increased infectivity in human cells does not significantly affect the neutralization resistance of viruses with truncated gp41. As shown by provision of excess Env in trans, high Env content in virions may be critical for antibody-mediated neutralization (Yuste et al., 2005). Further studies will be required to understand the mechanism of resistance to neutralization conferred by gp41 truncation.

In the present study, we demonstrated that truncation of the cytoplasmic tail of gp41 contributes to resistance to antibody-mediated neutralization. Although non-human primate models of SIV infection are commonly used to estimate vaccine efficacy, the lack of broadly neutralizing MAbs has hampered development of antibody-based vaccine candidates in an SIV-macaque model. The broadly neutralizing MAb B404, which neutralizes multiple, diverse SIV isolates (Kuwata et al., 2013), is a useful tool for understanding the mechanism of neutralization in an SIV-macaque model and will contribute to the development of HIV-1 vaccines.

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Assessment, Diagnosis, and Treatment of HIV-Associated Neurocognitive Disorder: A Consensus Report of the Mind Exchange Program

The Mind Exchange Working Group

Many practical clinical questions regarding the management of human immunodeficiency virus (HIV)–associated neurocognitive disorder (HAND) remain unanswered. We sought to identify and develop practical answers to key clinical questions in HAND management. Sixty-six specialists from 30 countries provided input into the program, which was overseen by a steering committee. Fourteen questions were rated as being of greatest clinical importance. Answers were drafted by an expert group based on a comprehensive literature review. Sixty-three experts convened to determine consensus and level of evidence for the answers. Consensus was reached on all answers. For instance, good practice suggests that all HIV patients should be screened for HAND early in disease using standardized tools. Follow-up frequency depends on whether HAND is already present or whether clinical data suggest risk for developing HAND. Worsening neurocognitive impairment may trigger consideration of antiretroviral modification when other causes have been excluded. The Mind Exchange program provides practical guidance in the diagnosis, monitoring, and treatment of HAND.

Keywords. AIDS dementia complex; HIV-associated dementia (HAD); HIV-associated neurocognitive disorder (HAND); HIV encephalopathy; neurocognitive impairment.

Despite advances in the treatment of human immunodeficiency virus (HIV) [1], the central nervous system (CNS) is still often affected by this disease. Impairment of cognition caused by HIV disease is known as HIV-associated neurocognitive disorder (HAND) [2]. Importantly, compared with unaffected populations, HAND, even in its mild form, is associated with lower medication adherence [3], less ability to perform the most complex daily tasks [4–7], worse quality of life [8], difficulty obtaining employment, and shorter survival [8]. Athough the incidence of the most severe form of HAND—HIV-associated dementia (HAD)—has declined in the era of combination antiretroviral therapy (cART) [9], the incidence and prevalence of milder forms (asymptomatic neurocognitive impairment [ANI] and mild neurocognitive disorder [MND]) have remained stable or perhaps even increased [10]. In addition, as cART-treated patients survive into older age, there could be a rise in HAND due to interactive effects of chronic immune activation and aging on the CNS [11].

Gaps remain in translating emerging neuro-HIV research findings into clinical practice [12]. To address this problem, the Mind Exchange program was established with the goal to provide guidance of direct relevance to daily clinical practice. In this communication we describe the process of expert consensus development and specific recommendations on HAND diagnosis and management, based on the best available evidence.

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METHODS

Sixty-six specialists from a range of disciplines (including HIV clinicians, neurologists, neuropsychologists, clinical psychologists, and psychiatrists who care for and have experience with HIV patients) from 30 countries provided input into the Mind Exchange program, which took place between February 2011 and January 2012. The program was overseen by a steering committee of 5 experts, including 2 infectious disease specialists (from Italy and the United States), a neurologist (from Germany), a neuropsychiatrist (from the United States), and a clinical psychologist (from Spain).

The program comprised several stages (Figure 1). A broad list of clinical questions across the 5 topics (screening, diagnosis, monitoring, treatment/interventions, and prevention of HAND) was generated by a core group of international experts in a face-to-face meeting. A total of 83 questions were identified and included in a questionnaire for prioritization by the core expert group and a wider group of HIV clinicians; the questionnaire was circulated and returned by email, with 65 individuals from 30 countries responding. This process resulted in a final set of 14 questions identified as of critical clinical importance to be addressed during the remainder of the program.

A comprehensive literature search of PubMed and the Cochrane Library was performed for each of the 14 questions by a research or clinical fellow, or a member of the core expert group, using question-specific search strings and predefined limits (no time limit was specified). Abstracts from key international conferences were also searched.

For each question, a draft practical answer was generated by 2 or 3 members of the core expert group based on the findings of the literature review and their clinical opinion. Answers were reviewed by the steering committee and refined by the expert group. Following this, an international meeting with the steering committee, core expert group, and broader HIV clinician group was held to discuss and further refine the draft answers. These 63 participants from 30 countries voted on

Figure 1. Overview of the Mind Exchange program. Abbreviation: KOL, key opinion leader.

their level of agreement with each draft answer using a scale of 1–9 (where 1 = strong disagreement and 9 = strong agreement). Consensus was defined as at least 75% of participants scoring within the 7–9 range. If <75% of participants scored within this range, the answer was debated and revised, followed by a second vote. Similar voting methodology has been employed in development of other consensus-based guidelines in the United Kingdom [13, 14].

The core expert group then further refined the answers to improve clarity and to reduce their length for this document. No substantive changes in the content or meaning of the answers were made. A level of evidence and grade of recommendation was assigned to each statement in the final answers, in accordance with the Oxford Centre for Evidence-Based Medicine (CEBM) 2009 criteria [15]. This system covers all study types and is appropriate for assigning levels of evidence across the broad range of clinical questions.

RESULTS

The 14 key questions are presented in Table 1. Agreement was achieved on the draft answers to all 14 questions at the international meeting. Here we present a summary of the major points of the guidance derived from each of the answers to the 14 questions.

Screening for HAND

It is appropriate to assess neurocognitive functioning in all patients with HIV (CEBM 5; grade of recommendation [GOR] D) as there is limited rationale for screening only symptomatic patients (CEBM 2b) [16–19] or only those with recognized risk factors for HAND (eg, nadir $CD4^+$ T-cell counts <200 cells/μL) (CEBM 2b; GOR C) [20]. Furthermore, because the CNS is commonly one of the first targets of HIV infection, good practice suggests that a patient's neurocognitive profile should be assessed early (within 6 months of diagnosis, as soon as clinically appropriate) using a sensitive screening tool (CEBM 5; GOR D) [21]. If possible, screening should take place before the initiation of cART (CEBM 5; GOR D), as this will establish accurate baseline data and allow for subsequent changes to be more accurately assessed.

Although there are insufficient data to establish the best time for follow-up assessments (CEBM 2b) [22], the consensus group agreed that screening for HAND should occur every 6–12 months in higher-risk patients or every 12–24 months in lower-risk patients (CEBM 5; GOR D). Several risk factors (Table 2) have been independently associated with an increased likelihood of HAND. The clinical significance of risk factors should be considered in light of the patient's full medical history. Screening should also be carried out immediately if there is evidence of clinical deterioration (CEBM 5, GOR D) or at the time of major changes in clinical status (eg, cART initiation or change or diagnosis of mental health disorders; CEBM 3b; GOR C) [23].

Many brief screening approaches have been proposed for the detection of neurocognitive disorders; the benefits and limitations of those tools for which there is substantial literature on their use in HAND are presented in Table 3. In addition to paper-based tools, some computerized tools are also available for screening (eg, CogState [34]; CANTAB reaction time [35]). No single tool is suitable for use across all practice settings, and the choice of a HAND screening tool depends on a number of considerations, including the availability of a clinician suitably trained to administer and interpret each tool; whether the clinician wants to screen for HAD only or for the milder forms of HAND; the financial and time cost of testing; and the characteristics of the population in which the tool will be used (CEBM 5; GOR D). Neurocognitive screening tools

Table 1. Fourteen Key Clinical Questions That Were Identified and Addressed During the International Program

1	Which patients should be screened for HAND, and when? How often should patients be screened?
2	How can physicians identify patients at greater risk of HAND?
3	Which tools should be used to screen for HAND?
4	Which comorbidities should be considered in a patient with HAND?
5	How can HAND be differentiated from neurodegenerative diseases in older patients?
6	How should neuropsychological testing be approached in the diagnosis of HAND?
7	In addition to cognitive testing, which other assessments should be used in the diagnosis of HAND (eg, psychiatric assessment, lumbar puncture/CSF analysis, imaging, exclusion of other pathologies)?
8	What is the role of lumbar puncture/CSF analysis in the management of HAND, and when should it be performed?
9	When, and how often, should neurocognitive performance be reviewed in patients who have been diagnosed with HAND?
10	What is the natural history of ANI and MND, and how should this impact patient management?
11	What interventions should be considered in treated patients with persistent or worsening NCI and CSF viral load <50 copies/mL (nondetectable)? Should the ARV still be changed when the virus is not detectable in the CSF?
12	What is the risk of ARV-related neurotoxicity? What should be done if ARV neurotoxicity is suspected?
13	When/how should pharmacological agents other than ARV be used in the management of HAND?
14	What can be done to prevent HAND?

Abbreviations: ANI, asymptomatic neurocognitive impairment; ARV, antiretroviral; CSF, cerebrospinal fluid; HAND, human immunodeficiency virus–associated neurocognitive disorder; MND, mild neurocognitive disorder; NCI, neurocognitive impairment.

Table 2. Comorbidities and Risk Factors Important to the Identification and Differential Diagnosis of HIV-Associated Neurocognitive Disorder

Evidence-supported risk factors	Risk Factor/Comorbidity for HAND and/or Non-HIV-Related NCI	Can Assist Identification of Patients			CEBM Levels (See Question Details for References)
		With Current HAND	At Risk of Developing HAND in Future	At Risk of Non-HIV-Related NCI	
Readily assesiable in clinic					
Disease factors	Low nadir CD4$^+$ T-cell count	X	X		CEBM 1b
	High plasma HIV RNA; high CSF HIV RNA	X	X		CEBM 2b
	Low current CD4 (pre-cART)	X	X		CEBM 2b
	Presence of past HIV-related CNS diseases	X	X		CEBM 1b
	Longer HIV duration	X	X		CEBM 2b
Treatment factors	Low cART adherence	X	X		CEBM 1b
	Episodes of cART interruption	X	X		CEBM 2a
	Nonoptimal cART regimen	X	X		CEBM 2a
	Short cART duration (related to treatment failure)	X	X		CEBM 1b
Comorbidities	Positive HCV serostatus with high HCV RNA	X	X	X	CEBM 1b
	History of acute CV event			X	CEBM 1b
	CV risk factors (hyperlipidemia, elevated blood pressure, chronic diabetes, and diabetes type II)			X	CEBM 1/2b
	Anemia and thrombocytopenia	X	X	X	CEBM 1/2b
Demographic factors	Older age	X	X	X	CEBM 1b
	Low level of educational achievement	X	X	X	CEBM 2b
	Ethnicity	X	X	X	CEBM 2b
	Sex (female, as associated with lower socioeconomic status in some countries)	X	X	X	CEBM 3a
	Lack of access to standard care; poverty	X	X	X	CEBM 3b
Other neurological and psychiatric factors	Neuropsychiatric disorders, eg, MDD, anxiety, PTSD, psychosis, bipolar disorder (current or history of)	X	X	X	CEBM 2b
	Illicit drug/alcohol abuse/dependence (current or history of)	X	X	X	CEBM 2a
	Syphilis or systemic infection	X	X	X	CEBM 2b
	Alzheimer's disease			X	Use APA (in press)
	Cerebrovascular disease			X	Use APA (in press)
	Traumatic brain injury and seizure	X	X	X	CEBM 2b
	Vitamin or hormone deficiency			X	Use APA (in press)
	Prior HCV coinfection[a]			X	CEBM 2b
Complex cART factors	Lower CPE	X	X		CEBM 2a
	cART neurotoxicity			X	CEBM 3b
Difficult to assess in clinic					
Biomarkers	Abnormal CSF neopterin	X			CEBM 2a
	Abnormal plasma HIV DNA	X			CEBM 2b
	Abnormal NFL	X			CEBM 2a
	Abnormal MCP–1	X			CEBM 2a
	Abnormal serum osteopontin	X			CEBM 4

Abbreviations: APA, American Psychiatric Association *Diagnostic and Statistical Manual of Mental Disorders* (in press; see www.dsm5.org); ARV, antiretroviral; cART, combined antiretroviral therapy; CEBM, Centre for Evidence-Based Medicine; CNS, central nervous system; CPE, central nervous system penetration efficiency; CSF, cerebrospinal fluid; CV, cardiovascular; DNA, deoxyribonucleic acid; HAND, HIV-associated neurocognitive disorder; HCV, hepatitis C virus; HIV, human immunodeficiency virus; MCP-1, monocyte chemoattractant protein-1; MDD, major depressive disorder; NCI, neurocognitive impairment; pts, patients; NFL, neurofilament light chain protein; PTSD, posttraumatic stress disorder; RNA, ribonucleic acid.

[a] Evidence of previous HCV infection (ie, in HCV-infected patients with no active HCV RNA, and without liver cirrhosis or failure) should also be considered a risk factor for non-HIV-related NCI [2]. For full referencing of this table please see the Supplementary Data.