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R5X4 Viruses Are Evolutionary, Functional, and Antigenic Intermediates in the Pathway of a Simian-Human Immunodeficiency Virus Coreceptor Switch▽

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To examine the pathway of the coreceptor switching of CCR5-using (R5) virus to CXCR4-using (X4) virus in simian-human immunodeficiency virus $SHIV_{SF162P3N}$-infected rhesus macaque BR24, analysis was performed on variants present at 20 weeks postinfection, the time when the signature gp120 V3 loop sequence of the X4 switch variant was first detected by PCR. Unexpectedly, circulating and tissue variants with His/Ile instead of the signature X4 V3 His/Arg insertions predominated at this time point. Phylogenetic analysis of the sequences of the C2 conserved region to the V5 variable loop of the envelope (Env) protein showed that viruses bearing HI insertions represented evolutionary intermediates between the parental $SHIV_{SF162P3N}$ and the final X4 HR switch variant. Functional analyses demonstrated that the HI variants were phenotypic intermediates as well, capable of using both CCR5 and CXCR4 for entry. However, the R5X4 intermediate virus entered CCR5-expressing target cells less efficiently than the parental R5 strain and was more sensitive to both CCR5 and CXCR4 inhibitors than either the parental R5 or the final X4 virus. It was also more sensitive than the parental R5 virus to antibody neutralization, especially to agents directed against the CD4 binding site, but not as sensitive as the late X4 virus. Significantly, the V3 loop sequence that determined CXCR4 use also conferred soluble CD4 neutralization sensitivity. Collectively, the data illustrate that, similar to human immunodeficiency virus type 1 (HIV-1) infection in individuals, the evolution from CCR5 to CXCR4 usage in BR24 transitions through an intermediate phase with reduced virus entry and coreceptor usage efficiencies. The data further support a model linking an open envelope gp120 conformation, better CD4 binding, and expansion to CXCR4 usage.

Entry of human immunodeficiency virus type 1 (HIV-1) into target cells requires the CD4 receptor and one of two coreceptors, CCR5 or CXCR4 (2). CCR5-using (R5) virus predominates early in infection, but in about 50% of subtype B-infected individuals, CXCR4-tropic (X4) virus appears and coexists with R5 viruses, and this is associated with more rapid decline of $CD4^+$ T cells and poorer prognosis (3, 5, 11, 12, 58, 66). The basis for X4 emergence late in infection remains ill defined, but among the hypotheses proposed are mutation by chance, CCR5 bearing target cell limitation, and differential immune recognition of X4 and R5 viruses (43, 53). Furthermore, it is unclear whether X4 viruses evolve during the course of infection or were present at time of transmission but preferentially suppressed early in infection.

In HIV-1-infected individuals and in tissue culture systems, the pathway to coreceptor switching transitions through intermediates with the ability to use CXCR4 in addition to CCR5 (12, 50, 57, 60, 61). Compared to the early or inoculating R5 viruses, these R5X4 dual-tropic viruses often display a loss in replicative fitness as well as less efficient use of the CCR5 coreceptor in vitro (30, 50). It has been suggested that the fitness disadvantage of the intermediates compared with the initial R5 virus constitutes one of the blockades to coreceptor switching, explaining the late appearance of X4 viruses (50). Additionally, recently emerged R5X4 and X4 viruses in humans are found to be more sensitive to antibody neutralization than coexisting R5 viruses, implicating antiviral antibody response as another obstacle to coreceptor switching (6).

We recently described the first case of a coreceptor switch in rhesus macaque BR24 that was infected with the late R5 simian-human immunodeficiency virus $SHIV_{SF162P3N}$ isolate (23). Animal BR24 progressed to disease rapidly after transient seroconversion. Virus recovered at end-stage disease (28 weeks postinfection) was shown to use CXCR4 exclusively and, compared to the inoculating virus, was highly susceptible to antibody neutralization, in particular, to agents such as soluble CD4 (sCD4) and the monoclonal antibody (MAb) immunoglobulin G1b12 (IgG1b12) directed at the CD4 binding site (CD4BS). Furthermore, similar to cases reported in humans (10, 46), X4 emergence lagged rather than preceded or coincided with the onset of a precipitous $CD4^+$ T-cell decline in macaque BR24, lending support to the notion that X4 emergence is the result, rather than the cause, of immune failure. The goal of the present study is to reconstruct the pathway to coreceptor switching in macaque BR24 and determine the consequences for envelope (Env) protein functions associated with evolution to CXCR4 usage. We seek to identify transitional intermediates and to assess the costs and benefits of, and reasons for, coreceptor switching in a nonhuman primate model of HIV/AIDS.

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MATERIALS AND METHODS

Cells. 293T cells and TZM-bl cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin, streptomycin, and L-glutamine. The latter expressed CD4, CCR5, and CXCR4 and contained integrated reporter genes for firefly luciferase and β-galactosidase under control of the HIV-1 long terminal repeat. U87 glioma cell lines stably expressing CD4 and one of the chemokine receptors were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, antibiotics, 1 μg/ml puromycin (Sigma-Aldrich, St. Louise, MO), and 300 μg/ml G418 (geneticin; Invitrogen, Carlsbad, CA).

RNA/DNA extraction, sequencing, and analysis. Viral RNA was prepared from 500 μl of BR24 week 20 plasma using a commercially available RNA extraction kit (Qiagen, Chatsworth, CA), and reverse transcribed with Superscript III RT (Invitrogen) and random hexamer primers (Amersham Pharmacia, Piscataway, NJ). For DNA extraction, cells from axillary lymph node (LN) biopsy sampled at 20 weeks postinfection (wpi) or from tissues collected at necropsy were used. The V1 to V5 region of gp120 was amplified from the reverse transcription products or extracted proviral DNA by *Taq* DNA polymerase (Qiagen) with primers ED5 and ED12 or ES7 and ES8 as previously described (14). PCR products were cloned with the TOPO TA cloning kit (Invitrogen) per the manufacturer's instructions, followed by direct automated sequencing of cloned gp120 amplicons (SeqWright; Fisher Scientific, Houston, TX). Nucleotide sequences were aligned using the CLUSTAL X program, version 1.81, and further adjusted manually (28).

Env gp160 subcloning and pseudotype virus production. The construction of Env expression plasmids for the inoculating R5 $SHIV_{SF162P3}$ (EnvP3N), the final X4 $SHIV_{BR24N}$ (EnvBR24N), and EnvP3N expressing the V3 variable loop of EnvBR24N [EnvP3N(HR-V3)] has been previously described (23). For expression of the full-length gp160 coding sequence of viruses predominating at 20 wpi in macaque BR24 ($EnvHI_{20}$, week 20 virus bearing the HI insertion in Env gp160), DNA was extracted from LN cells, amplified with primers SH52 (5′-TAG ATC GAA TTC TAG AGC CCT GGA AGC ATC CAG GAA GTC AGC CTA-3′) and SH53 (5′-AGA GAG GGA TCC TCC AGT CCC CCC TTT TCT TTT AAA AA-3′), and subcloned into the pCAGGS expression plasmid. To generate P3N recombinant Env carrying the V3 loop of $EnvHI_{20}$, PCR-based overlapping extension methodology was employed (21). Briefly, with EnvP3N serving as a template for PCR amplification, the outer primers used were SH43 (5′-AAGACAGAATTCATGAGAGTGAAGGGGATCAGGAAG-3′) and SH44 (5′-AGAGAGGGATCCTTATAGCAAAGCCCTTTC AAAGCCCT-3′), and the inner primers were SH66 (5′-AATAATACAAGAAAAAGTATACGTATA CATATAGGACCGGGGAGAGCATTTTATGC-3′) and SH67 (5′-TATATG TATACGTATACTTTTTCTTGTATTATTGTTAGGTCTTGTACAATTAAT TTCTAC-3′), which encompassed the amino acid changes found in the V3 loop of $EnvHI_{20}$ (underlined). The resulting amplified fragment was verified by sequencing and subcloned back into pCAGGS. Single-cycle replication-competent luciferase reporter viruses were prepared by transfecting 293T cells with the NL4.3-Luc-E^- R^+ vector and the corresponding Env expression plasmid with polyethylenimine (Polyscience, Warrington, PA). Supernatants were harvested 72 h posttransfection and quantified for $p24^{Gag}$ antigen content by antigen capture assay (Alliance HIV-1 p24 antigen enzyme-linked immunosorbent assay kit; Perkin Elmer, Waltham, MA).

Entry and blocking assays. Single-cycle infectivity assays were used to measure the entry efficiency of various Env proteins. Briefly, 10^4 U87.CD4.CCR5 or U87.CD4.CXCR4 cells were seeded in 96-well plates 24 h before use. Cells were infected with 5 ng of a $p24^{Gag}$-equivalent of the indicated pseudotyped viruses, followed by incubation for 72 h at 37°C. At the end of the incubation period, cells were harvested, lysed, and measured for luciferase activity according to the manufacturer's instructions (Luciferase Assay System; Promega, Madison, WI). Entry, as quantified by luciferase activity, was measured with a Dynex MLX microtiter plate luminometer (Dynex Technologies, Inc., Chantilly, VA). For the entry-blocking assays, U87.CD4 indicator or TZM-bl cells were pretreated with various concentrations of PSC-RANTES (gift of Oliver Hartley, University of Zurich), TAK-779, or AMD3100 for 30 min at 37°C before infection. The percentage of entry blocking was calculated by the amount of entry in the presence of the inhibitor relative to that in the absence of the inhibitor, and 50% and 90% inhibitory concentrations (IC_{50} and IC_{90}, respectively) were determined using Prism 4 software (GraphPad, San Diego, CA).

Neutralization assay and antibodies. Virus neutralization sensitivity was assessed using TZM-bl or U87.CD4 indicator cells in 96-well plates. Briefly, equal volumes (50 μl) of pseudotyped viruses (5 ng of $p24^{Gag}$ equivalent) and serial dilutions of heat-inactivated macaque serum or MAbs were incubated for 30 min at 37°C and then added to cells, in duplicate wells, for an additional 2 to 3 h at 37°C. A total of 100 μl of medium was then added to each well, and the virus-MAb cultures were maintained for 72 h. At the end of the culture period, the cells were lysed and processed for β-galactosidase (Galacto-Star System; Applied Biosystems, Bedford, MA) or luciferase activity. A neutralization curve was generated by plotting the percentage of neutralization versus serum or MAb dilution, with IC_{50} and IC_{90} values calculated as described above. Control cultures received virus in the absence of antibodies. The human MAb IgG1b12 was kindly supplied by Dennis Burton (Scripps Research institute, La Jolla, CA), CD4-IgG2 (PRO 542) was from William Olsen (Progenics Pharmaceuticals, Tarrytown, NY), and the anti-V3 antibody 447-52D was from Susan Zolla-Pazner. MAbs 2G12, 4E10, and 2F5 were obtained through the NIH AIDS Research and Reference Reagent Program.

RESULTS

Variants with HI insertions predominate at the time of first X4 virus appearance. We recently reported that mutations within the gp120 V3 loop, notably an insertion of two basic amino acids (HR) immediately upstream of the tip of the GPGR crown of the R5 $SHIV_{SF162P3N}$ virus, were responsible for the change in coreceptor preference of the virus replicating in infected rhesus macaque BR24 at the time of death (23). Variants bearing HR sequences were first detected by PCR in the plasma and peripheral blood mononuclear cells of BR24 at 20 wpi, 8 weeks prior to euthanasia. To determine the spectrum of X4 variants at the time of their first emergence, as well as to reconstruct the evolutionary pathway to the phenotypic switch from R5 to X4 in an SHIV model, the Env V1 to V5 sequences of week 20 circulating viruses were cloned, determined, and compared. V3 loop sequence analysis showed that the predominating virus (22 of 29 clones sequenced) had HI instead of HR insertions, with a net positive charge of +7 or +8 instead of +9 for this gp120 region (Fig. 1A). In contrast to the final X4, but similar to the initial R5 virus, the highly conserved potential N-linked glycosylation (PNG) site at the base of the V3 loop was intact for all 29 sequences of week 20 Env examined. Variants with HI insertions also predominated in the axillary LN sampled at week 20. Of 41 Env clones obtained from this tissue site, 35 harbored HI insertions, 4 had HR insertions, while the remaining 2 clones retained wild-type (WT) R5 sequences. One of the 35 HI-bearing clones from the axillary LN lacked the V3 PNG site.

The tissue reservoirs of viruses with HI or HR insertion sequences were further examined using samples obtained at necropsy (week 28). Results showed that these sequences were poorly represented in the gut (intraepithelial and laminar propria lymphocytes [LPL]) and intestinal LNs (colonic and mesenteric LNs) but predominated in peripheral LNs such as the axillary, iliac, and inguinal LNs (Fig. 1B). Within the latter tissue sites, variants with HR insertions now dominated over those with HI insertions. For example, of 43 Env clones sequenced from the axillary LNs at time of death, 31 harbored HR insertions, 10 had WT sequences, and only 2 contained HI insertions. The change in dominance over time from variants with HI to those with HR insertions in axillary LNs suggests that the former are less fit than the late X4 variants.

HI-bearing variants are intermediates in the evolutionary pathway from CCR5 to CXCR4 use. Neighbor-joining phylogenetic analysis of sequences of Env V1 to V5 supports a progressive mutational process leading to phenotypic switching in macaque BR24, with HI-bearing sequences present at 20 wpi representing evolutionary intermediates to HR-bearing

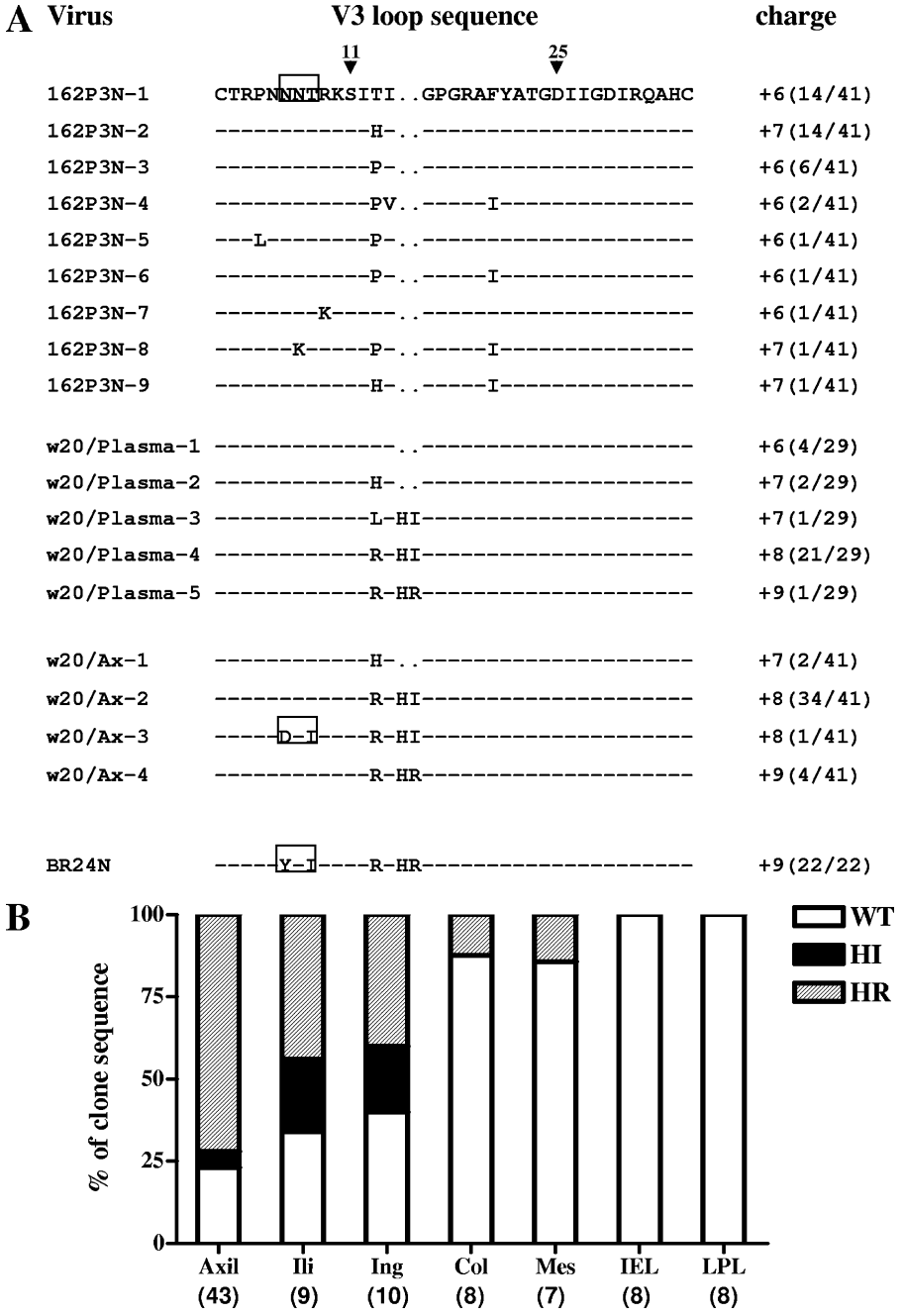

FIG. 1. (A) Comparison of V3 loop sequences of the parental R5 $SHIV_{SF162P3N}$ isolate, the recovered X4 virus $SHIV_{BR24N}$, and viruses present in plasma and axillary (Ax) LN of macaque BR24 at 20 wpi. Dashes denote similarity in sequences, and gaps are indicated as dots. The net positive charge of this region is indicated in the right column. Positions 11 and 25 within the V3 loop that frequently distinguished HIV-1 X4 and R5 viruses (16) are designated by arrowheads, and the highly conserved PNG site at the V3 base is boxed. The numbers in parentheses represent the number of clones with the indicated V3 loop sequence reported relative to the total number of clones sequenced. (B) Representation of SHIV viral variants without (WT) or with HI or HR insertions in various tissue sites of macaque BR24 at the time of death (28 wpi). Percentages of Env clones present in axillary (axil), iliac (ili), inguinal (ing), colonic (col), and mesenteric (mes) LNs, as well as intraepithelial lymphocytes (IEL) and LPL of the gut with the indicated signature V3 loop sequences are shown. Numbers in parenthesis indicate the number of gp120 clones sequenced from each of the tissue sites.

sequences (Fig. 2A). Although minor week 20 viral populations sitting in various locations in the tree were noted, it is clear, based on the genetic distance, that the closest relatives of $SHIV_{BR24N}$ sequences were among the week 20 sequences. We previously reported that, compared to the inoculating R5 $SHIV_{SF162P3N}$ isolate, the final X4 $SHIV_{BR24N}$ variant harbored nine unique amino acid changes in V2 and V3 and in a C4 region of gp120 implicated in CCR5 binding (23). gp120 sequence of viruses bearing HI insertions differed from $SHIV_{SF162P3N}$ as well as $SHIV_{BR24N}$ in five of the nine amino

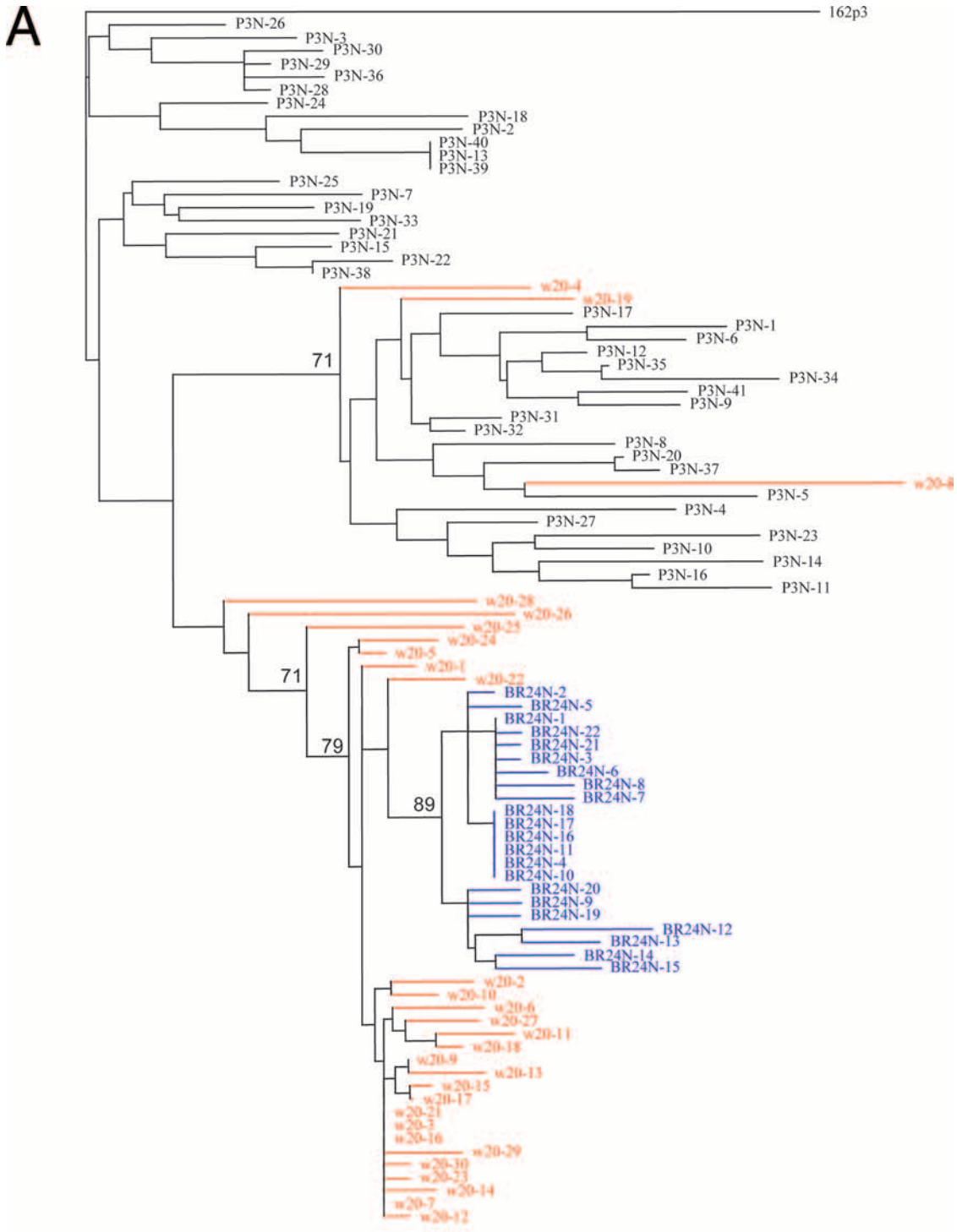

FIG. 2. (A) Phylogenetic tree showing the relationship between the Env V1 to V5 sequences of the parental R5 $SHIV_{162P3N}$, the week 20 variants, and the final X4 $SHIV_{BR24N}$. A neighbor-joining tree was generated, with the $SHIV_{SF162P3}$ sequence serving as an outgroup. Week 20

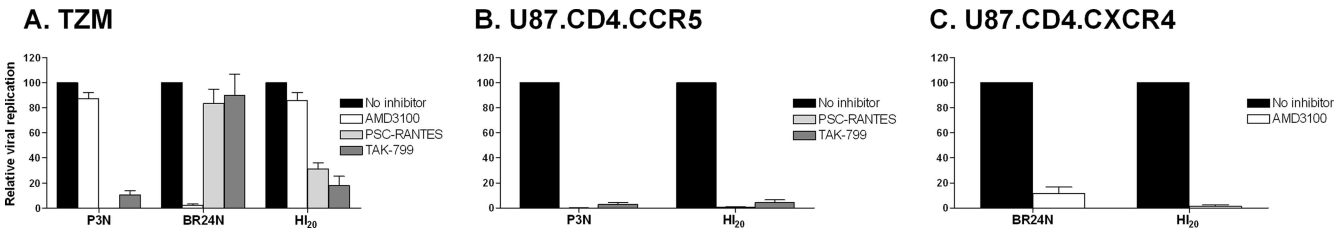

FIG. 3. Coreceptor usage of predominant week 20 HI insertion-bearing viruses. (A) CCR5 and CXCR4 usage of viruses pseudotyped with Env of $SHIV_{SF162P3N}$ (P3N), $SHIV_{BR24N}$ (BR24N), and week 20 viruses bearing HI insertions (HI_{20}) was determined by entry inhibition into TZM-bl $CCR5^+$ $CXCR4^+$ (TZM) cells with 1 μM CXCR4 inhibitor (AMD3100) or CCR5 inhibitors (TAK-779 and PSC-RANTES). Infection and blocking with cognate coreceptor inhibitors were also performed in the U87.CD4.CCR5 (B) and U87.CD4.CXCR4 (C) cells. Error bars indicate standard errors of the mean of data in triplicate wells. Results shown are representative of at least two independent experiments.

acids (Fig. 2B). In addition, the HI variants differed from both the parental R5 and final X4 SHIV virus in the V5 hypervariable region, where a charge substitution (change of G to E) and repositioning of a PNG site (change of T to N) were noted. Besides the V3 loop (17, 20), several regions of gp120, including the V1-V2 loop (9, 17, 29, 72), are known to influence coreceptor choice. Furthermore, the C4 domain interacts with the V3 loop (44, 74) and influences the binding and fusion steps of the entry process (63). Combined, the data show that viruses predominating at week 20 are evolutionary intermediates, differing from the initial R5 SHIV and final X4 variant in Env regions known to modulate the efficiency of coreceptor use.

Variants with HI insertions use both CCR5 and CXCR4 for entry. To determine the tropism of intermediate week 20 viruses bearing the HI insertions, the predominant HI Env gp160 sequences (designated $EnvHI_{20}$) were used to generate pseudotype viruses and tested for coreceptor usage. Infection with viruses bearing the Env of the parental R5 $SHIV_{SF162P3N}$ (EnvP3N) and the final X4 variant $SHIV_{BR24N}$ (EnvBR24N) served as controls. In single-round infectivity assays, infection of TZM-bl cells that express both coreceptors with virus pseudotypes bearing $EnvHI_{20}$ was efficiently blocked by the CCR5 inhibitors TAK-779 and PSC-RANTES (~80%) but only minimally by the CXCR4 antagonist AMD3100 (~20%), mostly likely because the virus is using the CCR5 coreceptor in these cells for entry (Fig. 3A). Infection and blocking experiments in the U87.CD4 indicator cells, however, demonstrated the dual-tropic nature of HI_{20} Env-bearing viruses, which were able to infect cells expressing the CCR5 (Fig. 3B) or CXCR4 coreceptor (Fig. 3C) and were potently inhibited by cognate coreceptor inhibitors in their respective indicator cell lines. Thus, HI variants demonstrated the typical properties of a dual-tropic virus, while the later HR-bearing variants used almost exclusively the CXCR4 coreceptor. Variants with HI insertions in the V3 loop, therefore, are functional intermediates as well as evolutionary ones.

R5X4 intermediates have reduced entry efficiency into CCR5-expressing cells and use CCR5 and CXCR4 less efficiently. To assess the costs and benefits of coreceptor switching in rhesus macaques, we examined the entry and coreceptor usage efficiencies of the inoculating R5, R5X4 intermediate, and final X4 viruses by infectivity and dose titration entry-blocking experiments. Results of single-round infectivity assays showed that $EnvHI_{20}$ mediated similar entry into CXCR4-expressing cells compared to EnvBR24N but showed a 1.5 log lower entry into CCR5 expressing cells than the parental R5 EnvP3N (Fig. 4A). Substitution of the V3 loop of $EnvHI_{20}$ into EnvP3N generated a recombinant EnvP3N(HI-V3) that now mediated entry only in CXCR4-expressing cells, albeit much less efficiently than the parental dual-tropic HI_{20} and the final X4 Envs, indicating that mutations outside of the V3 loop of the R5X4 intermediate virus contribute to coreceptor usage and virus entry efficiencies. Increased sensitivity to antagonists of both coreceptors was also observed for viruses expressing $EnvHI_{20}$. Concentrations of the CCR5 inhibitor PSC-RANTES needed to suppress infection of the R5X4 intermediate were fourfold less than those required to inhibit the parental R5 virus (IC_{50}s of 0.6 nM and 2.4 nM, respectively) (Fig. 4B). Similarly, sensitivity to inhibition with the CXCR4 blocker AMD3100 was enhanced 4.5-fold compared to the final X4 virus (IC_{50}s of 71 nM and 323 nM, respectively) (Fig. 4C). The findings of less efficient entry into CCR5-expressing cells and increased sensitivity to CCR5 and CXCR4 blockers for R5X4 viruses suggest a loss of competitive fitness of these dual-tropic intermediates compared to the parental R5 and the final X4 viruses.

R5X4 variants are neutralization-sensitive antigenic intermediates. We showed previously that the final X4 $SHIV_{BR24N}$ virus was more sensitive to antibody-mediated neutralization than the parental R5 $SHIV_{SF162P3N}$ virus, particularly to CD4-IgG2 and MAb IgG1b12 that are directed against the CD4BS (7, 23, 77). To determine whether the R5X4 intermediates present at 20 wpi share this characteristic with the final virus

plasma sequences are marked in red while those of the X4 $SHIV_{BR24N}$ isolate are in blue. The scale bar indicates genetic distances along the horizontal branches. The values on the branches represent the percentage of bootstrapped trees out of 1,000 replicates. Sequence gaps are excluded for analysis. (B) Comparison of the predicted V2, V3, C4, and V5 gp120 amino acid sequences of $SHIV_{SF162P3N}$, $SHIV_{BR24N}$, and week 20 Envs. The nine amino acids in X4 $SHIV_{BR24N}$ that differed from R5 $SHIV_{SF162P3N}$ Env gp120 are indicated in red. Dashes denote similarity in sequence, dots indicate gaps, and the PNG sites are boxed. Residues that differ between the viruses are numbered based on their relative positions in the HXB2 sequence. w, week.

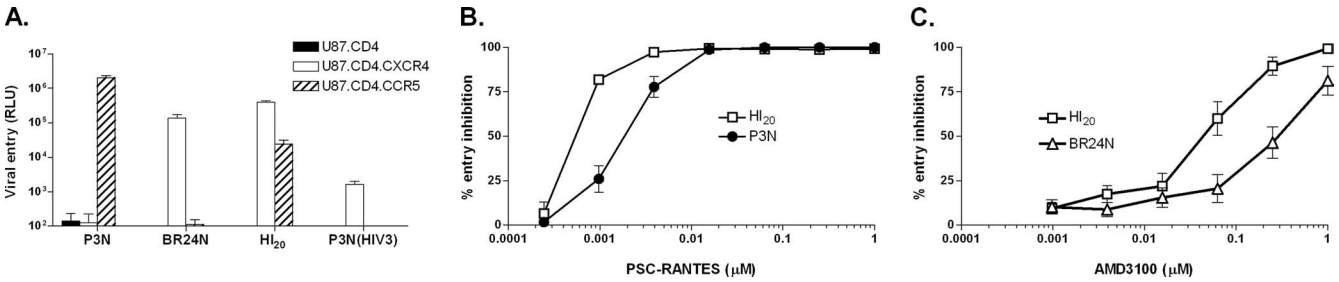

FIG. 4. Entry and coreceptor usage efficiencies of R5, R5X4, and X4 SHIV viruses. (A) Relative entry into U87.CD4 (closed bars), U87.CD4.CXCR4 (open bars), and U87.CD4.CCR5 (hatched bars) cells of luciferase reporter virus expressing Env of R5 $SHIV_{SF162P3N}$ (P3N), X4 $SHIV_{BR24N}$ (BR24N), R5X4 (HI_{20}), and V3 loop recombinant EnvP3N(HI-V3). (B) Blocking of P3N (●) and R5X4 (□) virus entry into U87.CD4.CCR5 cells with increasing concentrations of the CCR5 inhibitor PSC-RANTES. (C) Blocking of BR24N (△) and R5X4 (□) virus entry into U87.CD4.CXCR4 cells with increasing concentrations of the CXCR4 inhibitor AMD3100. Data shown are means ± standard errors of the mean of at least three independent experiments. RLU, relative light units.

that uses only CXCR4, as well as to assess the antigenic relatedness of the viral Envs, the neutralization profile of these viruses to serum from macaques infected with the parental R5 $SHIV_{SF162P3N}$ and the final X4 variant $SHIV_{BR24N}$ (23) was first determined. Results showed that $EnvHI_{20}$ and EnvBR24 virus pseudotypes were equally sensitive to neutralization with antibodies present in the R5 SHIV serum and more so than viruses bearing the parental R5 EnvP3N. Ninety percent neutralization of the CXCR4-using viruses was achieved at a serum dilution of 1:1,500, but a similar degree of neutralization of the R5 virus required a 30-fold higher serum titer. Furthermore, while the parental R5 virus was resistant to neutralization with serum antibodies from an X4 SHIV-infected macaque, the R5X4 virus was efficiently (90%) neutralized at a dilution of 1:120, and the final X4 virus was the most sensitive of all, with 90% neutralization at a serum dilution of 1:750 (Fig. 5A). The finding that $EnvHI_{20}$ can be differentiated from EnvBR24 in terms of neutralization sensitivity to the X4 SHIV serum implies differences in their antigenic structures.

Neutralization with several well-defined broadly neutralizing MAbs was performed to better understand the mechanism underlying enhanced sensitivity to neutralization of the R5X4 intermediate virus to SHIV-positive macaque serum. Soluble CD4 (CD4-IgG2) neutralized the two CXCR4-using viruses with equal potency, achieving 50% neutralization at concentrations that were 40-fold lower than those required to neutralize the parental R5 virus (IC_{50}s of 0.05 and 0.07 μg/ml for the R5X4 and X4 viruses, respectively, compared to 2.2 μg/ml for the R5 virus). While the parental virus was resistant, the R5X4 virus was sensitive to neutralization with the CD4BS MAb IgG1b12 but with an IC_{50} that was twofold higher than the final X4 virus (IC_{50}s of 0.15 and 0. 075 μg/ml, respectively) (Fig. 5B). Both CXCR4-using viruses but not the parental R5 virus were potently neutralized by the V3 loop MAb 447-52D (IC_{50} of 0.004 μg/ml). In contrast, no difference in sensitivity of the three viruses to 2G12 directed at sugar moieties of gp120 was observed. Of the two MAbs directed against the membrane-proximal external region of gp41 tested, 4E10 moderately neutralized virus pseudotypes bearing the HI_{20} and BR24N Envs (IC_{50} of >1 μg/ml) but not P3N, while 2F5 neutralized the final X4 (IC_{50} of 0.4 μg/ml) and to some extent the parental R5 virus (IC_{50} of 3 μg/ml), but the R5X4 virus was resistant. A K-to-N mutation in the 2F5 epitope of the dual-tropic virus accounted for its resistance (data not shown). Taken together, the results showed that the R5X4 intermediate shares a generalized increased sensitivity to neutralization with the final X4 virus but differs from the final X4 virus due to its reduced sensitivity to X4 SHIV serum and to IgG1b12 neutralization. The finding of enhanced neutralization susceptibility of the dual-tropic and the X4 viruses to agents directed at the CD4BSs and the V3 loop compared to the parental R5 virus suggests adoption of an increasingly open conformation of the Env gp120 of viruses in macaque BR24 over time, with exposure of both the CD4 and coreceptor binding sites. The data further support the hypothesis proposed by us (23) and others (6, 36) that lowered or absent antibody-mediated selective pressure is one of the factors favoring the emergence of neutralization-sensitive, CXCR4-using variants.

CXCR4 usage and sCD4 neutralization sensitivity are functionally linked. CXCR4 usage and sCD4 neutralization sensitivity have been reported to be regulated by similar mechanisms via the V3 domain (27, 47), with the conserved 301N glycosylation site in the V3 base playing a particularly important role (35, 38, 51). To examine whether the increase in sCD4 neutralization sensitivity of the R5X4 and X4 SHIV viruses is functionally linked to their ability to use the CXCR4 coreceptor, the neutralization profile of R5 $SHIV_{SF162P3N}$ derivatives that contained the V3 loop of the R5X4 or X4 SHIV virus to CD4-IgG2 was examined. These derivatives, designated P3N(HI-V3) and P3N(HR-V3), respectively, altered the tropism of $SHIV_{SF162P3N}$ from CCR5 to CXCR4 use (Fig. 4A) (23). Results showed that the CXCR4-using V3 loop recombinant viruses displayed an sCD4 neutralization profile comparable to that observed with either the R5X4 or X4 SHIV virus (Table 1). Thus, mutations in V3 loop of the R5X4 intermediate and the final X4 SHIV variant that confer CXCR4 use are also sufficient to determine sCD4 neutralization sensitivity.

DISCUSSION

The characterization of SHIV variants present in a macaque at the time of X4 virus emergence provides further insight into the process of coreceptor switching. We find that viruses capable of using both coreceptors predominated in plasma and lymphoid tissues of macaque BR24 at week 20 but represented

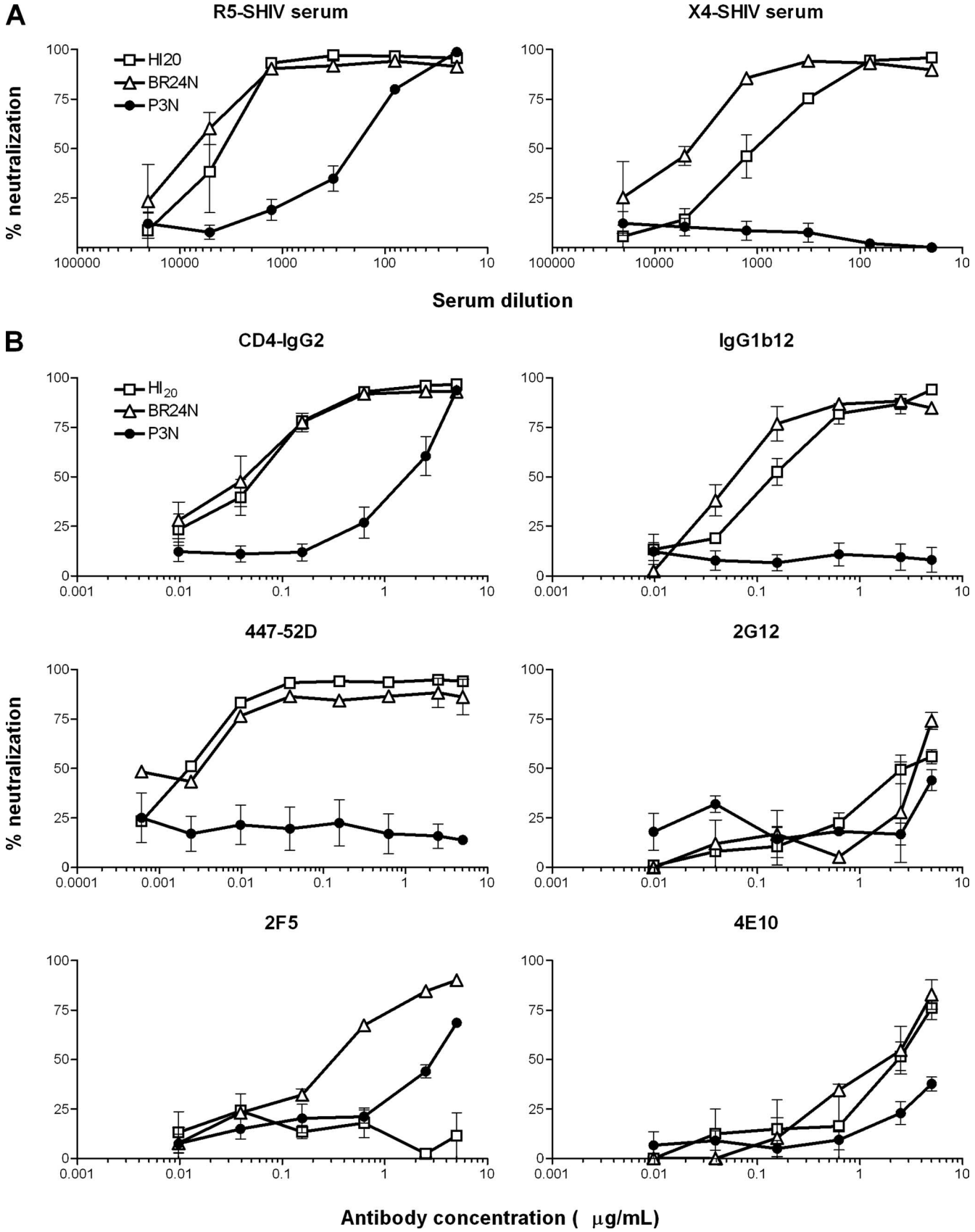

FIG. 5. Neutralization sensitivity of R5, R5X4, and X4 SHIVs. The neutralization susceptibility of the parental R5 $SHIV_{SF162P3N}$ (●), the final X4 $SHIV_{BR24N}$ (△), and the R5X4 intermediate (□) virus to antibodies in serum from R5 $SHIV_{SF162P3N}$-infected (R5-SHIV serum) and X4 $SHIV_{BR24N}$-infected (X4-SHIV serum) macaques (A) and to soluble CD4 (CD4-IgG2) and MAbs IgG1b12, 447-52D, 2G12, 2F5, and 4E10 (B) was determined in TZM-bl cells as described in Materials and Methods. Data shown are the means and standard errors of at least three independent neutralization experiments.

TABLE 1. CXCR4 usage and sCD4 neutralization sensitivity are functionally linked[a]

ENV type	Replication of virus on:		sCD4 neutralization (IC_{50} [μg/ml] ± SEM)[b]
	U87.CD4.CCR5 cells	U87.CD4.CXCR4 cells	
P3N	Yes	No	2.170 ± 0.103
HI_{20}	Yes	Yes	0.021 ± 0.004
P3N(HI-V3)	No	Yes	0.043 ± 0.001
BR24	No	Yes	0.038 ± 0.000
P3N(HR-V3)	No	Yes	0.025 ± 0.002

[a] The coreceptor preference and CD4-IgG2 neutralization susceptibility (concentrations achieving IC_{50}) of virus pseudotypes bearing the envelopes of the parental R5 $SHIV_{SF162P3N}$ (P3N), the week 20 R5X4 intermediate SHIV (HI_{20}), the final X4 $SHIV_{BR24N}$ (BR24N), and $SHIV_{SF162P3N}$ derivatives containing the V3 loop of the R5X4 [P3N(HI-V3)] or the final X4 [P3N(HR-V3)] SHIV are shown.

[b] Values represent three independent neutralization experiments.

only a minor population at the time of necropsy 8 weeks later. The dual-tropic viruses occupied an intermediate position between the parental R5 and final X4 SHIV virus in the phylogenetic tree, differing from these viruses in Env gp120 sequence regions known to modulate receptor and coreceptor binding. In vitro studies showed that R5X4 SHIV viruses were compromised in the efficiency of use of each of the coreceptors but shared Env conformations with both the parental R5 and X4 SHIV viruses, as probed by sensitivity to antibody neutralization. Thus, dual-tropic viruses represent temporal, evolutionary, functional, and antigenic intermediates in the pathway to coreceptor switch in rhesus macaques.

At necropsy, dual-tropic and X4 viruses predominated in peripheral LNs (axillary, iliac, and inguinal) but not in intestinal LNs (colonic and mesenteric) or LPL isolated from the jejunum or plasma. Memory $CD4^+$ T lymphocytes are enriched in the gut-associated lymphoid tissue (GALT) and have much higher CCR5 expression than lymphocytes from blood and LNs (22, 69). Accordingly, the GALT is an early site of R5 virus replication and massive destruction of $CD4^+$ T cells in humans and in macaques (4, 18, 19, 39, 40, 68). CCR5 expression levels have also been shown to be higher on $CD4^+$ T cells in the mesenteric LN than in the axillary LN (69). Thus, intestinal LNs could provide optimal sites for R5 virus replication and pathogenesis following the depletion of susceptible target cells in the gut. In this regard, our finding that X4 viruses, which evolved late in infection, reside principally in the peripheral lymph nodes suggests that a significant proportion of $CD4^+$ target T cells in the gut and intestinal LNs express both CCR5 and CXCR4, such that prior seeding and depletion of these CCR5/CXCR4-coexpressing target cells by R5 viruses limited the establishment of niches by the emerging X4 viruses at these sites. Alternatively, higher levels of stromal cell derived factor 1, the ligand of CXCR4, could be present in the GALT and intestinal LNs. Interestingly, a recent report in pediatric HIV infections indicates that the thymus or secondary lymphoid tissues may also play an important role in the evolution/amplification of coreceptor variants (55). Evolution and localization of X4 variants in peripheral lymphoid organs which are not frequently sampled could explain the observation of X4 emergence seen in only a subset of patients progressing to AIDS. Tissue data are very limiting for HIV-1 infection of humans, highlighting the usefulness of the SHIV model in providing a detailed picture of X4 evolution over time and in different tissue compartments of the host.

The dual-tropic SHIV intermediate virus entered CCR5-expressing cells less well and was more sensitive to entry inhibition with the CCR5 analog PSC-RANTES than the parental R5 virus. It was also more sensitive than the final X4 SHIV to inhibition with the CXCR4 inhibitor AMD3100. Correlation between diminished coreceptor usage efficiency and enhanced sensitivity to neutralization with coreceptor inhibitors of HIV-1 variants has been reported (48, 52) although in the case of AMD3100 differences in sensitivity could be due to differences in V3 loop sequence that influence the overall conformation of gp120 (15). Increased sensitivity to PSC-RANTES is also a robust indicator of better patient outcome (64), suggesting that increased sensitivity to CCR5 antagonists may also be related to reduced viral fitness. Accordingly, our findings indicate that the R5X4 intermediates in macaque BR24 have reduced replicative capacity and decreased CCR5 and CXCR4 binding efficiency. HIV-1 R5X4 intermediates have also been found to have reduced fitness (49, 50, 67) and display greater sensitivity to CCR5 antagonists and lower binding affinity to CCR5 than R5 viruses (1, 57, 59, 76). Decreasing sensitivity to CXCR4 antagonists of X4 HIV-1 variants that evolved during the natural course of infection has also been reported (62). Collectively, the data support a similar mechanistic basis for coreceptor switching in humans and macaques. Ongoing evolution of Env variants toward CXCR4 coreceptor usage in both rhesus macaques and humans is at the expense of CCR5 use, and R5X4 intermediate viruses are inefficient in engaging each of the chemokine receptors.

Why, then, does the switch from CCR5 to CXCR4 need to transition through an apparently weak and inefficient intermediate? It is conceivable that the intermediate arises by chance as a result of high virus replication and mutation rates. Indeed, the HI insertions seen in the V3 loop of the R5X4 intermediate SHIV could arise from copying error, resulting in gene duplication of the HI sequence immediately upstream of the GPGR crown in one of the major variants in the R5 SHIV inoculum (Fig. 1A, 162P3N-2). The mutations might become fixed because they confer the selective advantage of CXCR4 use and, hence, target cell expansion. Furthermore, at this stage of the infection within the host, R5X4 viruses may, in fact, be more fit than coexisting X4 viruses. The transient rise in viremia that accompanied the appearance of these intermediates at 20 wpi in macaque BR24 suggests that they are indeed fit, and preliminary data indicate that compared to the R5X4 intermediate, CXCR4-using variants that harbor HR insertions present at 20 wpi are poor in mediating viral entry (data not shown). Lastly, we found a small but consistent difference in neutralization susceptibility of the CXCR4-using SHIVs to serum antibodies and IgG1b12, with the R5X4 intermediate being more resistant than the X4 variant. Thus, the possibility exists that in the presence of even minimal residual humoral immune pressure, perhaps localized at tissue sites that were not sampled in this study, replication of a more neutralization-resistant virus like the HI insertions bearing intermediate and not the highly sensitive final X4 SHIV will be favored. Coreceptor switching was recently observed in a second R5 $SHIV_{SF162P3N}$-

infected macaque (24). It will be of interest to determine whether R5X4 intermediate viruses are also present in this second animal and whether similar costs and benefits are associated with switching via a dual-tropic intermediate.

Compared to the parental R5 virus, the dual-tropic and X4 viruses were highly susceptibility to neutralization with sCD4 and the anti-V3 loop antibody 447-52D, but, as noted above, the final X4 virus was twofold more sensitive than the R5X4 intermediate to neutralization with the CD4BS MAb IgG1b12. Structural studies showed that the CD4BS is recessed on the virion surface, shielded by the V1/V2 loop and associated carbohydrate structures (8, 33, 34, 37, 56, 71, 72, 75). The V1/V2 loop also masks the coreceptor binding site on gp120 that is composed of parts of the V3 loop and a surface formed by the bridging sheet (13, 25, 54, 73). Higher sensitivity of viruses to neutralization with sCD4, IgG1b12, and anti-V3 loop antibodies implies greater exposure of the CD4BS and chemokine receptor binding site that are usually sequestered away from the humoral immune response (32, 34), indicative of a more open Env conformation. Enhanced neutralization susceptibility of the CXCR4-using viruses to MAb directed against epitopes in gp41 lends further support to an open Env configuration of these viruses. The observation that the final X4 SHIV virus is more sensitive than the R5X4 intermediate to neutralization with SHIV serum and IgG1b12 suggests that there is greater exposure of the receptor binding site on the virus that uses only CXCR4 than on viruses that are capable of interacting with both coreceptors. We show that acquisition of CXCR4 use and sCD4 neutralization sensitivity of the R5X4 and X4 SHIV viruses are mediated by similar mechanisms. Furthermore, X4 emergence in BR24 (23) and in another $SHIV_{SF162P3N}$-infected macaque (24) lags rather than precedes the precipitous drop in $CD4^+$ T cells. In humans, CXCR4 use is associated with a lower CD4 cell count (5, 26, 41, 45, 65, 70), and X4 viruses are frequently recovered from rapid progressors (31, 42). Thus, it is tempting to speculate that adoption of a more open Env conformation, driven perhaps by the need to bind CD4 efficiently as the number of $CD4^+$ target T cells and/or amount of the receptor molecule on target cells becomes limited, will also facilitate interaction with CXCR4 but will, at the same time, reduce protection from antibody-mediated neutralization.

In conclusion, as in HIV-1 infection, evolution from CCR5 to CXCR4 in $SHIV_{SF162P3N}$-infected rhesus macaque BR24 transitions through an R5X4 intermediate phase characterized by reduced entry capacity and less efficient coreceptor usage in vitro. Similar to the final X4 virus, the dual-tropic intermediate SHIV is sensitive to sCD4 and anti-V3 loop antibody neutralization. This, coupled with the finding that sCD4 neutralization sensitivity and CXCR4 use are regulated by similar mechanisms, supports a link between open Env configuration, increased CD4 binding, better CXCR4 use, and enhanced humoral immune recognition that merits further investigation. R5 $SHIV_{SF162P3N}$ infection of rhesus macaques, in which the precise timing, tissue sites, and cause for change in Env glycoprotein structure and coreceptor preference can be studied, provides a valid and invaluable animal model to further elucidate the reasons for coreceptor switching.

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