Neutralization-Sensitive R5-Tropic Simian-Human Immunodeficiency Virus SHIV-2873Nip, Which Carries env Isolated from an Infant with a Recent HIV Clade C Infection (vol 83, pg 1422, 2009)

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Volume 83, no. 3, p. 1422–1432, 2009. SHIV-2873Nip was erroneously classified as a tier 1 virus. The correct neutralization sensitivity of this virus is tier 2, as demonstrated by replicate experiments. Throughout the article, SHIV-2873Nip should be referred to as a virus with tier 2 neutralization sensitivity.

Page 1422, Abstract, line 13: “high sensitivity” should read “moderate sensitivity.”
Page 1422, Abstract, line 14: “tier 1” should read “tier 2.”
Page 1428, column 2, line 4 from the bottom: “tier 1” should read “tier 2.”
Page 1428: Table 2 should appear as shown below.

Page 1430, column 1, paragraph 1, last line: “tier 1” should read “tier 2.”
Page 1430, column 2, paragraph 3, line 1: “tier 1” should read “tier 2.”
Page 1430, column 2, paragraph 3, last line: “tier 1” should read “tier 2.”
Page 1431, column 1, paragraph 1, line 5: “tier 1” should read “tier 2.”
Page 1431, column 1, paragraph 2, line 2: “tier 1” should read “tier 2.”

TABLE 2. Neutralization sensitivity of R5 SHIV strains carrying HIV clade B or C env to soluble CD4, human NMAbs, and serum samples

<table>
<thead>
<tr>
<th>HIV strain</th>
<th>ICso in TZM-bl cells*</th>
<th>Reciprocal serum dilution</th>
<th>Tier</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Soluble CD4</td>
<td>IgG1b12</td>
<td>2G12</td>
</tr>
<tr>
<td>SHIV-2873Nip (Zambian env; early isolate)</td>
<td>C</td>
<td>1.5</td>
<td>&gt;25</td>
</tr>
<tr>
<td>SHIV-1157ipd3N4 (Zambian env; late isolate)</td>
<td>C</td>
<td>0.4</td>
<td>7.0</td>
</tr>
<tr>
<td>SHIVSF162P4</td>
<td>B</td>
<td>0.2</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>SHIVSF162P3</td>
<td>B</td>
<td>11.6</td>
<td>6.0</td>
</tr>
</tbody>
</table>

* Values represent the concentration (μg/ml for soluble CD4 and human NMAbs IgG1b12, 2G12, 2F5, and 4E10, and HIVIG) or the dilution (for serum samples) at which relative luciferase units were reduced 50% compared to virus control wells. BB47, BB55, BB68, BB75, BB80, BB81, and BB87 are serum samples from individuals infected with HIV-1 clade C. The data shown are representative of the results of one of two replicate experiments.
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Human immunodeficiency virus clade C (HIV-C) accounts for >56% of all HIV infections worldwide. To investigate vaccine safety and efficacy in nonhuman primates, a pathogenic, R5-tropic, neutralization-sensitive simian-human immunodeficiency virus (SHIV) carrying HIV-C env would be desirable. We have constructed SHIV-2873Nip, an R5-tropic SHIV carrying a primary pediatric HIV-C env gene isolated from a 2-month-old Zambian infant, who died within 1 year of birth. SHIV-2873Ni was constructed using SHIV-1157ipd3N4 (R. J. Song, A. L. Chenine, R. A. Rasmussen, C. R. Ruprecht, S. Mirshahidi, R. D. Grisson, W. Xu, J. B. Whitney, L. M. Goins, H. Ong, P. L. Li, E. Shai-Kobiler, T. Wang, C. M. McCam, H. Zhang, C. Wood, C. Kankasa, W. E. Secor, H. M. McClure, E. Strobert, J. G. Else, and R. M. Ruprecht. J. Virol. 80:8729–8738, 2006) as the backbone, since the latter contains additional NF-κB sites in the long terminal repeats to enhance viral replicative capacity. The parental virus, SHIV-2873Ni, was serially passaged through five rhesus monkeys (RMs): SHIV-2873Nip, the resulting passaged virus, was reisolated from the fourth recipient about 1 year postinoculation. SHIV-2873Nip was replication competent in RM peripheral blood mononuclear cells of all random donors tested and was exclusively R5 tropic, and its env gene clustered with HIV-C by phylogenetic analysis; its high sensitivity to neutralization led to classification as a tier 1 virus. Indian-origin RMs were inoculated by different mucosal routes, resulting in high peak viral RNA loads. Signs of virus-induced disease include depletion of gut CD4+ T lymphocytes, loss of memory T cells in blood, and thrombocytopenia that resulted in fatal cerebral hemorrhage. SHIV-2873Nip is a highly replication-competent, mucosally transmissible, pathogenic R5-tropic virus that will be useful to study viral pathogenesis and to assess the efficacy of immunogens targeting HIV-C Env.

Currently, 33 million people are living with human immunodeficiency virus (HIV)/AIDS (www.unaids.org), and the majority of them live in sub-Saharan Africa and South and Southeast Asia, including China and India, where HIV subtype C (HIV-C) circulates in >90% of the HIV-infected population (UNAIDS) (50). This distribution makes HIV-C the most prevalent subtype in the global pandemic, accounting for >56% of all HIV infections worldwide (www.unaids.org). Globally, HIV is one of the leading causes of childhood morbidity and mortality. Children account for 20% of all HIV-related deaths, 7% of individuals living with HIV, and 16% of new infections annually (reviewed in references 26, 29, and 38). In sub-Saharan Africa, HIV-C is responsible for approximately 50% of all infections, and a significant number of infections are in infants and children. HIV transmission from infected mothers to their infants is the primary mode of infection in children and can occur in utero, intrapartum, or postnatally through breast milk. The use of antiretroviral drugs has successfully reduced the rate of HIV infection in infants in the developed world to approximately 1%; nevertheless, such regimens have only recently become available in many of the developing nations where mother-to-child transmission of HIV is most significant (reviewed in references 26 and 38).

Simian-human immunodeficiency viruses (SHIVs) are chimeric viruses that contain HIV envelope genes in the simian immunodeficiency virus (SIV) backbone. They have been used in a wide range of studies investigating lentiviral pathogenesis, antiviral immunity, virus-host interactions, mucosal transmission, and vaccine and drug efficacy (20). However, the majority of current SHIV strains utilize envelope genes derived from HIV clade B strains, which represent fewer than 10% of all global infections. Therefore, the available SHIV chimeras do not reflect the genetic diversity of the HIV epidemic, which is dominated by non-B clades, especially by HIV-C. Only a few studies have focused on developing anti-clade C Env vaccines (25, 27, 44, 49), with one efficacy study in primate models (44). To investigate lentiviral pathogenesis as well as anti-HIV-C vaccine safety and efficacy in nonhuman primate models, a
pathogenic, CCR5-restricted, clade C SHIV (SHIV-C) would be very useful.

Previously, we have generated an R5-tropic SHIV-C, SHIV-1157i (6, 51), which carries env from a 6-month-old Zambian infant born to an HIV-positive mother. During prospective long-term follow-up, this infant turned out to be a long-term nonprogressor who has remained asymptomatic at 8 years of age (61). The rhesus monkey (RM)-adapted strain, SHIV-1157ip, was pathogenic and has caused AIDS in several monkeys thus far, but with a relatively low rate of disease progression. AIDS developed in RMs between 127 and 300 weeks postinoculation (17a). A late virus was reisolated and engineered to contain extra NF-kB sites in the long terminal repeats (LTRs) (51); follow-up times for monkeys infected with this late form are not yet sufficient to assess development to AIDS, although signs of disease have developed. A possible explanation is that the env gene used to construct the original SHIV-1157i is an important determinant of the disease progression rate. The fact that the env gene was derived from a long-term nonprogressor may be linked to the relatively slow disease progression we observed in RMs infected with SHIV carrying the corresponding env gene.

We sought to test whether constructing an R5-tropic SHIV with an env gene derived from a rapid progressesurfer would give rise to a more virulent R5-tropic SHIV-C. Although HIV- or SIV-infected individuals with either typical rates of disease progression or long-term non-progressor have been studied extensively, few reports were focused on the virologic and immunologic characteristics of patients with rapid disease progression (9, 22). Patients who progress to AIDS within 1 to 2 years from the time of infection have been identified among infants and adults (7, 13, 34, 35, 46), with a higher frequency in infant populations. These patients demonstrate rapid loss of CD4+ T cells and lack potent cellular and humoral immune responses.

Here we report the construction of SHIV-2873Ni, a chimera that carries env of an R5-tropic HIV- C strain isolated from a rapid progressor, a 2-month-old Zambian baby who died of AIDS-related disease within 1 year of birth. SHIV-2873Ni was serially passaged through five RMs; SHIV-2873Nip, the passaged virus, was reisolated and characterized from the fourth recipient about 1 year postinoculation when signs of disease were manifest. The RM-adapted virus caused T-cell depletion within a few months postinoculation.

MATERIALS AND METHODS

Original virus isolates and nomenclature. HIV-2873i is a biological isolate obtained from a Zambian infant at 2 months of age. The infant, born to an HIV-C-infected mother, was PCR negative at birth and rapidly progressed to AIDS-related death within 1 year. The designation “i” indicates a virus strain (or env gene) isolated from an infant. SHIV-2873Ni is the original, nonadapted infectious molecular clone that contains two NF-kB sites in the 3′ LTR instead of the usual single NF-kB site present in the SIVmac239 LTR. This duplicate NF-kB site is copied into the 5′ LTR during subsequent reverse transcription steps of the retroviral life cycle (8). SHIV-2873Nip, a biological isolate obtained after passage of SHIV-2873Ni through four RMs, was reisolated from a monkey systemically infected for approximately 1 year; “p” designates a passaged (or monkey-adapted) virus.

Cell lines, antibodies, and viruses. U87 or GHOST cell lines, which express CD4 only or CD4 with different chemokine receptors, as well as CEM.NKR.CCR5 cells, were provided by the NIH AIDS Research and Reference Reagents Program (ARRRP) (GermanLandtown, MD). Neutralizing monoclonal antibodies (NMAbs) 2F5 (36), 2G12 (56), and 4E10 (54) were provided by Hermann Katinez (Pomuny Science, Vienna, Austria). MAb b12 (1) is an immunoglobulin (IgG1) isotype and was produced by expression in recombinant CHO cells (kindly provided by Dennis Burton, Scripps Research Institute, La Jolla, CA). CEMx174-GFP cells, provided by Barbara Felber (National Cancer Institute, Frederick, MD), contain the green fluorescent protein (GFP) gene under HIV type 1 (HIV-1) LTR regulation and express CXCR4 but not CCR5. TZM-bl cells (also called FCS-3bl [clone 13] cells; ARRRP) (11) are derived from a HeLa cell line (JC-53) that stably expresses CD4 and CCR5. TZM-bl cells also express luciferase and β-galactosidase under the control of the HIV-1 LTR.

Animals and animal care. RMs (Macaca mulatta) of Indian origin were used in this study. The animals were kept according to National Institutes of Health guidelines on the care and use of laboratory animals at the Yerkes National Primate Research Center (YNPRC) (Emory University, Atlanta, GA). These facilities are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. Animal experiments were approved by the Animal Care and Use Committees of Emory University and the Dana-Farber Cancer Institute.

Construction of SHIV-2873Ni molecular clones. Peripheral blood mononuclear cells (PBMC) from Zambian infant 2873i were collected 2 months after the infant was born to an HIV-positive mother (62) and were briefly co-cultured with normal human donor PBMC. DNA from this coculture was extracted for PCR amplification. A pair of specific primers, designed to amplify the entire HIV-1 env gene, incorporated the HindIII or XhoI restriction enzyme sites and had the following sequences: 2873-HindIII, 5′-GGGGGAACCTTATGAGGATGGTG GGGATACAGAGG-3′, and 2873-XhoI, 5′-CCCCCTCTAGTAATGGCAAAG GTCTCTTCTAGACC-3′. The full-length HIV-2873i env was digested with the restriction endonucleases HindIII and cloned into the HIV-1 LTR of a late-stage HIV-1 isolate (SHIV-1157ipd3 of Indian origin) using the KpnI-BamHI fragment of HIV-2873i (spanning most of gp120, the entire gp120 extracellular domain, the transmembrane region, and part of the cytoplasmic domain) was amplified to replace the corresponding region of SHIV-1157ipd3 env. The modified 3′ half of env was ligated with the 5′ half of env as a (23) proviral DNA to generate SHIV-2873Ni (51).

Coreceptor usage of SHIV constructs. The U87 or GHOST cell lines expressing CD4 alone or CD4 and HIV-1 or SIV coreceptors were used to study virus tropism. U87,CD4; U87,CD4,CCR1; U87,CD4,CCR2; U87,CD4,CCR3; U87,CD4,CCR5; U87,CD4,CCR5, GHOST,BOB, and GHOST.BONZO were infected with virus stock. Cells were washed and resuspended in 1 ml of fresh medium. On days 0, 2, 4, and 6, supernatants were collected for p27 titration.

PCR and sequencing analysis. Chromosomal DNA was extracted from 105 PBMC from animal RN-9 using a DNeat genomic DNA isolation kit (Molec-ular Research Center Inc., Cincinnati, OH). To analyze the molecular evolution of SHIV-2873Ni env during in vivo passage, two different primers were synthesized to amplify the entire env gene (approximately 2.5 kb) of SHIV-2873Ni isolated from the last animal, RN-9, about 1 year postinoculation, after it had developed signs of disease. The env gene of SHIV-2873Ni was amplified using the following primers: forward (5′-CCCCCAACCTTACCCACATGAGATGAAA GGAGAAATACTC-3′) and reverse (5′-CCCCGGAATTCCTCCTTCTCCTATATACCTCC-3′); the PCR was carried out under end point dilution conditions. The amplified fragment was cloned into the HindIII and EcoRI sites of pCDNA/mye-His B vector for sequencing. Eight clones carrying an infectious env gene were randomly picked for DNA sequencing.

Phylogenetic analysis. The sequences of the env genes of SHIV-2873Ni, SHIV-2873Nip, other clade C SHIVs generated by us (SHIV-1157i, SHIV-1157ip, and SHIV-1157ipd3), and HIV1084i (14) were aligned with full-length reference sequences of several group M viruses obtained from the Los Alamos sequence database (http://hiv.lanl.gov/content/hiv-db/SUBTYPE_REF/align.html). Nucleo-tide sequences were gap-stipped and aligned using CLUSTAL X (55), and neighbor-joining trees were generated with the Kimura two-parameter substitution model.

VOL. 83, 2009 NEUTRALIZATION-SENSITIVE PEDIATRIC R5 CLADE C SHIV 1423
The neutralization sensitivity of SHIV-2873Nip was determined using both PBMC-based and TZM-bl reporter cell line-based neutralization assays, as described previously (11, 19, 24, 45). In both cases, serial dilutions of either RM sera or MAbs were set up in triplicate in 96-well plates, with virus and cells in well volume of 100 μl, and the experiment was repeated in triplicate. The RM PBMC-grown stock has a p27 concentration of 140 ng/ml and 4 × 10^6 50% tissue culture infectious doses (TCID₅₀) per ml as titrated in TZM-bl cells according to published protocols (11).

Neutralization assays. The neutralization sensitivity of SHIV-2873Nip was determined using both PBMC-based and TZM-bl reporter cell line-based neutralization assays, as described previously (11, 19, 24, 45). In both cases, serial dilutions of either RM sera or MAbs were set up in triplicate in 96-well plates, and virus was added (50 to 200 TCID₅₀) and incubated for 1 h at 37°C. Either PBMC or TZM-bl cells were then added. For assays employing immune sera, neutralization titers were calculated based on virus production in wells containing sera pooled from four naive RMs as negative controls. In both assays, the concentration of serum giving 50% neutralization of virus production (50% inhibitory concentration [IC₅₀]) was calculated using the level of virus production in control wells containing the same dilution of pooled naive sera; for MAb titers, the IC₅₀ was calculated based on control wells containing virus plus cells only.

For the PBMC-based assays, human PBMC were stimulated overnight with phytohemagglutinin (5 μg/ml), washed, and added to wells at 2 × 10⁶/well. In assays testing sera, PBMC were washed in assay plates after 1 day of culture, and fresh interleukin-2 (10 U/ml)-containing medium was added; alternatively, in PBMC-based assays testing MAbs, the MAbs were not washed away but were diluted 1:1 with fresh medium daily, starting on day 3 of the experiment. Because the latter assay condition takes into account the long half-lives of antibodies, neutralization titers may differ slightly from titers measured by other methods (2, 32).

Aliquots of supernatants were harvested every other day and assayed for p27 levels in wells containing only virus plus cells, and neutralization activity was measured on the culture day showing a linear phase of increase. For TZM-bl-based assays, cells were added in the presence of DEAE dextran (40 μg/ml) and washed once on day 1, Luciferase substrate (Bright-Glo, Promega, Madison, WI) was added on day 2, and luciferase activity was measured in a lumimeter.

Measurement of plasma viral RNA levels. Plasma viral RNA was isolated by the use of the QiaAmp viral RNA minikit (Qiagen), and viral RNA levels were measured by quantitative reverse-transcriptase PCR for SIV gag sequences (17) at weeks 0, 1, 2, 4, 8, and 8 and monthly thereafter. The assay sensitivity was 50 viral RNA copies/ml.

Oral and i.r. inoculation of SHIV-2873Nip. Indian-origin RMs received 3 ml or 1 ml of the large-scale virus stock by the oral or intrarectal (i.r.) route, respectively. Six additional animals were infected weekly by the oral route with 10⁴ TCID₅₀ (two monkeys) or 5,000 TCID₅₀ (four monkeys). Our protocol stipulates that animals remaining aviremic or failing to reach plasma viral RNA levels of ≥10 copies/ml at the 2-week time point after the fifth low-dose virus exposure would receive a single high-dose i.r. challenge (50,000 TCID₅₀). Blood was collected at 0, 1, 2, 4, 8, and 16 weeks and at 3-month intervals postinoculation to determine viral RNA loads and to measure T-cell subsets.

Isolation of cells from blood and rectal biopsies. PBMC were isolated using standard procedures; lymphocytes from rectal biopsies were obtained by digestion with collagenase followed by a separation step using Percoll gradients as described previously (58). Briefly, 10 to 20 pinch biopsies were collected in complete RPMI and washed two times with ice-cold Hank’s balanced salt solution. Biopsies were digested with collagenase type IV (Worthington, Lakewood, NJ) and DNase I (Roche, Indianapolis, IN), passed through needles of decreasing size (16, 18, and 20 gauge; five or six times with each needle), and filtered through a 100-μm filter. Cells were suspended in 35% Percoll in phosphate-buffered saline, underlaid with 60% Percoll, and centrifuged at 2,500 rpm for 30 min. Cells from the interface were collected, washed, and resuspended in complete RPMI for analysis.

Phenotypic analysis of T cells from blood and rectum. For T-cell subset analyses, approximately 1 × 10⁶ PBMC or lymphocytes from rectal biopsies were surface stained with the following MAbs (BD Pharmingen, San Jose, CA): anti-CD3 conjugated to Alexa 700 (clone SP34-2), anti-CD4 (clone 5G9), anti-CD8 conjugated to peridinin chlorophyll protein (clone L-200), and anti-CD95 conjugated to allophycocyanin (clone DX2). The following MAbs were used for eBioscience (San Diego, CA): anti-CD28 conjugated to PE (clone 30G12), anti-CCR5 conjugated to phycoerythrin (clone 3A9), and anti-CD45RA (clone ALB11). Following staining, cells were acquired using LSRII (BD Biosciences, San Jose, CA) and analyzed using FlowJo software (TreeStar, Inc., San Carlos, CA). Lymphocytes were identified based on scatter pattern, and CD3⁺ CD8⁻ CD4⁺ cells were considered CD4⁺ T cells, while CD3⁺ CD8⁺ CD4⁻ cells were considered CD8⁺ T cells. CD3⁺ CD4⁺ T cells were gated based on CD28 and CD95 expression to define memory CD4⁺ T-cell subpopulations: naive (CD28⁺ CD95⁻), central memory (CD28⁻ CD95⁺), and effector memory (CD28⁻ CD95⁺).

Statistical analysis. Statistical analysis was performed using Prism (GraphPad Software, version 4). Differences in percentages and numbers of lymphocytes in groups of uninfected versus SHIV-2873Nip-infected animals were compared using the Mann-Whitney test.

Nucleotide sequence accession number. The complete nucleotide sequence of the env gene of SHIV-2873Nip has been submitted to GenBank; the accession number is EU526392.
days 17 and 21. These data implied that despite the introduction of extra NF-κB binding sites into the LTRs to increase viral replicative capacity (51), the new SHIV strain still needed to undergo adaptation for optimal replication in RM.

Adaptation of SHIV-2873Ni to RMs and generation of SHIV-2873Nip. Rapid animal-to-animal passage of whole blood at the time of peak viremia (week 2 postinoculation) was used to adapt the new SHIV-C strain. This adaptation strategy selects viruses for improved replication fitness in the new host species without favoring neutralization escape variants, since neutralizing antibody responses typically take many weeks to mature. SHIV-2873Niwas passaged in five Indian-origin RMs (Fig. 3A). The initial cell-free SHIV-2873Ni viral stock was prepared in RM PBMC that were exposed to cell-free supernatant of 293T cells transfected with proviral DNA. The first macaque, RBl-9, was inoculated intravenously with 10 ml of SHIV-2873Ni stock; peak viremia reached $2.5 \times 10^5$ RNA copies/ml at week 2 postinoculation (Fig. 3B). Four additional animals were subjected to serial blood transfer (Fig. 3A), in which 10 ml of whole blood collected at week 2 postinoculation was directly transfused into recipients RAg-9, RAi-8, RWA-9, RNt-9, and RQc-9. Animal RWa-9 had previously been exposed to supernatant of 293T cells transfected with SHIV-2873Ni proviral DNA but had remained uninfected. After receiving infected blood from RM RAi-8, animal RWa-9 became infected, as did all monkeys enrolled in the serial virus passage described in Fig. 3A. All six animals seroconverted (data not shown). The passaged virus reached the highest peak viremia level in the last recipient, monkey RQc-9. We reisolated a virus about 1 year later from monkey RNt-9, the penultimate virus recipient in the adaptation schema. This animal was persistently viremic and had signs of disease progression at that time. The passaged virus, SHIV-2873Nip, is an uncloned biological isolate that was able to replicate in PBMC of 10 out of 10 RM donors (data not shown), indicating its adaptation to the new host species, although there was donor-to-donor variability in virus replication as expected for outbred animals.

Phylogenetic analysis. Primary full-length env genes were amplified from genomic DNA by PCR, cloned into the expression vector pcDNA6/B, and tested for infectivity. Infectious env genes of the various SHIV strains were sequenced, and phylogenetic analysis was performed using Clustal W and Paup. The env genes of the newly created SHIV strains clustered with HIV-C; among the strains tested, the closest relationship was found with our other set of SHIV-Cs derived from a pediatric HIV-C strain isolated from an infant of the same cohort of HIV-infected mothers/infants followed prospectively.

FIG. 1. Construction of SHIV-2873Ni. (A) Identification of an infectious env clone. Primary full-length env genes were PCR amplified from genomic DNA of normal human donor PBMC cocultured with infected PBMC of Zambian infant 2873i collected at 2 months of age and cloned into the expression vector pcDNA6/B. Infant 2873i was a rapid progressor who died of AIDS-related disease within 1 year of birth to an HIV-C-positive mother. To identify an infectious envelope, the resultant constructs were cotransfected into 293T cells with HIV-1 ΔEN, an infectious HIV provirus with deletions of env and nef and encoding GFP in lieu of nef. The resulting pseudovirus released into cell supernatants was used to infect CEM.NKR.CCR5 cells, which were screened for GFP expression. HIV-1 ΔEN cotransfected with ADA env was used as positive control. (B) Construction of SHIV-2873Nip. SHIV-1157ipd3N4 (51) was used as the backbone. The 2.2-kb KpnI (K)-BamHI (B) fragment of HIV-2873i (spanning most of gp120, the entire gp41 extracellular domain, the transmembrane region [TM], and part of the cytoplasmic domain) was amplified to replace the corresponding region of the proviral backbone. The modified 3′ half was ligated with the 5′ half of SHIV-vpu− proviral DNA to form full-length SHIV-2873Ni. NN, two NF-κB sites are present in the 3′ LTR; during viral replication, this duplication is copied into the 5′ LTR also.

FIG. 2. Coreceptor usage of SHIV-2873i, SHIV-2873Ni, and SHIV-2873Nip. U87.CD4.CXCR4 cells (A) and U87.CD4.CCR5 cells (B) were exposed to SHIV-2873Ni, SHIV-2873Nip, SHIVSF162 (HIV clade B env, R5), and SHIV-vpu− (HIV clade B env, X4). The levels of p27 Gag were measured in the supernatants as indicated. SHIV-2873i contains the standard SIVmac239 LTRs with only a single NF-κB site/LTR; it was built using SHIV-vpu− as the backbone.
at the University Hospital in Lusaka, Zambia. Thus, the proximity of the SHIV-2873Nip and SHIV-1157ipd3N4 env genes on the phylogenetic tree reflects the closeness of HIV-C strains circulating within the same community (Fig. 4A). During env evolution in the SHIV-infected RMs, the genes diverged as expected for chronically infected hosts (Fig. 4B).

**Evolution of SHIV-2873Nip Env during adaptation.** Sequence analysis of SHIV-2873Nip gp160, cloned from genomic DNA of RNt-9 PBMC collected at about 1 year after blood transfer, demonstrated a number of mutations. Compared with the parental Env sequence, SHIV-2873Ni, the SHIV-2873Nip consensus sequence revealed 14 point mutations throughout gp160, a 5-amino-acid (aa) deletion at the end of V4, and a 3-aa deletion at the beginning of the V5 region (Fig. 4B).

**SHIV-2873Nip exclusively uses CCR5 as coreceptor.** We assessed the coreceptor usage of SHIV-2873Nip as described for Fig. 2B. We observed productive infection only in the CCR5-expressing cell line, suggesting that SHIV-2873Nip maintained R5 tropism after rapid animal-to-animal passage.

**Susceptibility of SHIV-2873Ni and SHIV-2873Nip to neutralization by human NMAbs.** If SHIV-2873Nip is to become a useful tool to assess vaccine efficacy against HIV-C, maintaining a neutralization-sensitive Env structure will be important for its use as challenge virus. First, we determined the susceptibility of SHIV-2873Ni and the passaged SHIV-2873Nip to the broadly reactive human NMAbs IgG1b12, 2G12, 2F5, and 4E10 in human PBMC; the IC50s were compared to those of SHIV-1157ipd3N4 (45), an infectious molecular clade C SHIV clone created by our group earlier. These NMAbs recognize conserved epitopes on HIV gp120 and on the extracellular domain of gp41. IgG1b12 targets the CD4 receptor binding site (63), 2G12 recognizes conserved mannan residues on gp120 (48), and 2F5, and 4E10 recognize a coiled-coil region on gp41 that plays a crucial role during virus fusion with the cell membrane (41, 54, 64, 65).

SHIV-2873Ni and SHIV-2873Nip were effectively neutralized by IgG1b12, 2F5, and 4E10 but not by 2G12 (Table 1) in PBMC-based assays. The parental SHIV-2873Ni had a neu-
neutralization profile that was similar to that of SHIV-2873Nip. The IC_{50}s for SHIV-2873Ni and SHIV-2873Nip obtained with NMAbs IgG1b12, 2F5, and 4E10 were generally lower than those for SHIV-1157ipd3N4 (Table 1). Similar results were obtained using RM PBMC in the neutralization assays. We could not assess the neutralization sensitivity of SHIV-2873Ni in RM PBMC, since this parental, nonadapted virus did not replicate in the RM donor PBMC pool tested. Of note, none of the SHIV-Cs tested were susceptible to NMAb 2G12. Its epitope includes N-linked mannan moieties associated with the five residues N295, N332, N339, N386, and N392, with glycans attached to N295, N332, and N392 contributing to a core epitope (47, 48). Only four of these five residues are present in SHIV-2873Ni and SHIV-2873Nip Env sequences, and the crucial N295 residue was replaced by T295. The 2G12 epitope has been found to be missing in many primary HIV-C isolates (24). Compared with the linear motif NWFDIT recognized by 4E10, two amino acids (S versus D and S versus T) of this epitope were different in the predicted SHIV-2873Ni and SHIV-2873Nip Env sequences. In addition, these residues are not among the crucial residues of this epitope (64). With regard to the linear epitope ELDKWA recognized by 2F5, ALDSWN was found in both SHIV-2873Ni and SHIV-2873Nip with three residue substitutions compared to the standard epitope (4). The DSW motif, instead of DKW, may affect 2F5 binding efficiency.

FIG. 4. (A) Phylogenetic tree showing the relationship between SHIV-2873Ni and SHIV-2873Nip Env sequences and those of other primary strains of HIV. Phylogenetic trees were constructed from full-length Env sequences by using the neighbor-joining method. Major subtypes of HIV group M were used as reference sequences; sequences from SHIV-1157i, SHIV-1157ip, SHIV-1157ipd3N4 (51), and HIV1084i (14) were also included, since HIV1084i and the env genes in these SHIVs had been derived from the same cohort of infected mothers and their infants in Lusaka, Zambia. The scale bar indicates the genetic distance along the horizontal branches, and the numbers at the nodes are bootstrap values. (B) Evolution of SHIV-2873Nip Env during passage and replication in monkey Rnt-9. The deletions of 5 and 3 aa at the end and beginning of the V4 and V5 domains of gp120, respectively, in the adapted SHIV-2873Nip are shown. The Env consensus sequence for SHIV-2873Nip was derived by sequencing eight individual clones carrying infectious env genes.
Susceptibility of SHIV-2873Nip to neutralization by autologous and heterologous RM plasma/sera. To test the susceptibility of SHIV-2873Nip to neutralization by polyclonal antibodies, we performed a series of PBMC- and TZM-bl-based neutralization assays with autologous and heterologous RM plasma or serum samples. Autologous RM plasma samples from monkey RNt-9 were tested at the time of virus isolation and 10 months later. Virus was tested in the presence of plasma from naive controls and experimental animals; the ratio of the two values was used to calculate the percent inhibition. SHIV-2873Nip could be neutralized by autologous plasma (Table 1), and the NAb titers in the RM increased with time.

Sera from RM chronically infected with an earlier form of SHIV-1157ipd3N4 potently neutralized SHIV-2873Nip (Table 1 [data for only two RMs are shown]). Similar results were obtained using the TZM-bl neutralization assay (data not shown).

Neutralization tier assignment. Recently, a tier system has been developed to differentiate the neutralization sensitivities of primary HIV or SHIV strains (30). Tier 1 strains are noticeably neutralization sensitive; tier 2 strains are more difficult to neutralize and represent average sensitivity for primary isolates. To assess the neutralization sensitivity, we have tested SHIV-2873Nip against a panel of human NMAbs and polyclonal sera collected from HIV-positive individuals in TZM-bl cells. According to the data in Table 2, SHIV-2873Nip was classified as a tier 1 virus and our “late-stage” virus, SHIV-1157ipd3N4, falls into tier 2. SHIVSF162P3 and SHIV SF162P4, which had been classified previously as tier 2 and tier 1 viruses, respectively, were used for comparison in the assay.

### TABLE 1. Neutralization of clade C SHIV strains in human and RM PBMC

<table>
<thead>
<tr>
<th>Source of Abs (human NMAb or RM name)</th>
<th>SHIV-2873Ni</th>
<th>SHIV-2873Nip</th>
<th>SHIV-1157ipd3N4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human PBMC</td>
<td>Human PBMC</td>
<td>Human PBMC</td>
<td>Human PBMC</td>
</tr>
<tr>
<td>Human NMAbs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG1b12</td>
<td>0.36 ND</td>
<td>0.4</td>
<td>0.03</td>
</tr>
<tr>
<td>2G12</td>
<td>&gt;40 ND</td>
<td>&gt;40</td>
<td>&gt;40</td>
</tr>
<tr>
<td>2F5</td>
<td>0.32 ND</td>
<td>0.3</td>
<td>0.18</td>
</tr>
<tr>
<td>4E10</td>
<td>0.035 ND</td>
<td>0.035</td>
<td>0.05</td>
</tr>
<tr>
<td>4×b</td>
<td>0.40 ND</td>
<td>0.09</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Polyclonal RM plasma/sera

| Autologous plasma samples            |              |              |                 |
| RNt-9 (time of virus isolation)      | ND           | 662          | ND              |
| RNt-9 (10 mo after virus isolation)  | ND           | 1,036        | ND              |

Heterologous sera

| Heterologous sera                    |              |              |                 |
| RHy-9 (SHIV-1157ipd3N4-infected RM)  | ND           | 3,722        | ND              |
| RJa-9 (SHIV-1157ip-infected RM)      | ND           | 2,048        | ND              |

* Values are concentrations (μg/ml) for NMAbs IgG1b12, 2G12, and 4E10 or dilutions (for plasma or serum samples). All three viruses lack the 2G12 epitope. ND, not determined.

* 4×, quadruple combination of IgG1b12, 2G12, 2F5, and 4E10 at a 1:1:1:1 ratio.

* RHy-9 was described by Rasmussen et al. (44).

### TABLE 2. Neutralization sensitivity of R5-tropic SHIV strains carrying HIV clade B or C env to soluble CD4, human NMAbs, and serum samples

<table>
<thead>
<tr>
<th>SHIV strain</th>
<th>Clade</th>
<th>Soluble CD4</th>
<th>IgG1b12</th>
<th>2G12</th>
<th>2F5</th>
<th>4E10</th>
<th>HIV1G</th>
<th>BB47</th>
<th>BB55</th>
<th>BB68</th>
<th>BB75</th>
<th>BB80</th>
<th>BB81</th>
<th>BB87</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHIV-2873Nip (Zambian env; early isolate)</td>
<td>C</td>
<td>0.1</td>
<td>0.5</td>
<td>&gt;25</td>
<td>&gt;25</td>
<td>&gt;25</td>
<td>16.8</td>
<td>340</td>
<td>876</td>
<td>509</td>
<td>716</td>
<td>1,273</td>
<td>157</td>
<td>876</td>
</tr>
<tr>
<td>SHIV-1157ipd3N4 (Zambian env; late isolate)</td>
<td>C</td>
<td>0.4</td>
<td>7.0</td>
<td>&gt;25</td>
<td>&gt;25</td>
<td>&gt;25</td>
<td>1,160</td>
<td>105</td>
<td>131</td>
<td>86</td>
<td>79</td>
<td>72</td>
<td>47</td>
<td>260</td>
</tr>
<tr>
<td>SHIVSF162P4</td>
<td>B</td>
<td>0.2</td>
<td>&lt;0.01</td>
<td>15.7</td>
<td>1.5</td>
<td>0.7</td>
<td>25</td>
<td>363</td>
<td>2,543</td>
<td>328</td>
<td>615</td>
<td>828</td>
<td>211</td>
<td>360</td>
</tr>
<tr>
<td>SHIVSF162P3</td>
<td>B</td>
<td>11.6</td>
<td>6.0</td>
<td>&gt;25</td>
<td>&gt;25</td>
<td>&gt;25</td>
<td>1,505</td>
<td>24</td>
<td>65</td>
<td>22</td>
<td>27</td>
<td>25</td>
<td>&lt;20</td>
<td>180</td>
</tr>
</tbody>
</table>

* Values represent the concentration (μg/ml for soluble CD4 and human NMAbs IgG1b12, 2G12, 2F5, and 4E10 and HIV1G) or the dilution (for serum samples) at which relative luciferase units were reduced 50% compared to virus control wells. BB47, BB55, BB68, BB75, BB80, BB81, and BB87 are serum samples from individuals infected with HIV-1 clade C.
Mucosal transmissibility of SHIV-2873Nip. Since approximately 90% of all new HIV infections are acquired by mucosal exposure during sexual contact or via mother-to-child transmission, candidate AIDS vaccines should protect against mucosal virus challenge. Preclinical vaccine safety and efficacy studies in primate models should thus focus on mucosal virus challenges (reviewed in reference 59). We sought to test whether SHIV-2873Nip could be transmitted mucosally. A large stock of SHIV-2873Nip was generated in RM PBMC. To demonstrate mucosal transmissibility, we inoculated one monkey each by the oral and i.r. routes. Both animals showed robust viral replication during the first 2 weeks postinoculation (Fig. 5A). Next, we sought to determine whether SHIV-2873Nip could lead to systemic infection after repeated weekly low-dose i.r. challenges; we set five weekly inoculations as maximum. Of two RMs exposed to a weekly dose of 1,500 TCID50, one animal became viremic after three inoculations, whereas the second one did not and was subsequently given a single high dose of SHIV-2873Nip (30,000 TCID50). Viral loads were measured at the time points indicated. The horizontal dotted line indicates the lower limit of detection (<50 viral RNA copies/ml). (C and D) Estimation of CD4+ T-cell loss in gut (C) and blood (D), showing levels of CD4+ T cells in rectal biopsy specimens (collected between weeks 6 and 12 after the last, successful inoculation) and blood of RMs inoculated with SHIV-2873Nip compared with uninfected controls. The asterisk in panel C designates the percent CD4+ T cells in rectal mucosa of monkey RNt-9 collected at week 84 postinoculation.

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**Signs of SHIV-2873Nip pathogenicity.** Animal RNt-9, the penultimate recipient during serial passage, developed thrombocytopenia at 36 weeks postinoculation; the platelet count never recovered (Fig. 3C). At 92 weeks postinoculation, the monkey was found unresponsive with epistaxis, tachypnea, and an abnormal pulmonary exam by auscultation. At necropsy, severe multifocal cerebral hemorrhages were noted as well as extensive petechial ecchymotic hemorrhages involving the myocardium, stomach, liver, cecal mucosa, both lungs, and the bladder. The stomach also showed severe ulceration. Clearly, the monkey had developed a fatal thrombocytopenia. Although the absolute number of peripheral blood CD4+ T cells remained normal, the CD4+CD29- memory T-cell subset was persistently low from 26 weeks postinoculation onwards (Fig. 3D). Although most lymph nodes were normal at necropsy, splenomegaly was observed.

To assess the effect of SHIV-2873Nip on gut lymphocytes, all six of the mucosally inoculated RMs underwent rectal biopsies between weeks 6 and 12 postinfection; animal RNt-9 was subjected to rectal biopsy at week 84 postinfection (8 weeks before the fatal cerebral hemorrhage). We observed significant depletion of gut CD4+ T cells in all monkeys compared to uninfected controls (n = 7; P = 0.0006 by Mann-Whitney test) (Fig. 5C). In contrast, no statistically significant differences were noted in the blood (Fig. 5D); thus far, all of the SHIV-2873Nip-infected monkeys have maintained normal absolute CD4+ T-cell counts. However, four of the SHIV-2873Nip-infected animals (RWI-11, RAb-11, RTb-11, and RM-11) demonstrated loss of peripheral blood CD4+ memory T cells as assessed by CD4+CD29+ double staining (data not shown).
DISCUSSION

Here, we describe the development of a new R5-tropic SHIV-C that carries an African pediatric HIV-C env. The newly constructed molecular clone, SHIV-2873Ni, and the biological isolate, SHIV-2873Nip, have a number of relevant characteristics: (i) SHIV-2873Ni carries an HIV-C env from a pediatric rapid progressor; (ii) SHIV-2873Ni was cloned using the SHIV-1157ipd3N4 backbone, which has a deletion in the 3' end of HIV env that restored the original SIV env C terminus extending into nef; (iii) SHIV-2873Ni and its passaged progeny contain extra NF-kB sites; (iv) SHIV-2873Nip retained exclusive R5 tropism; (v) SHIV-2873Nip showed signs of pathogenicity (memory T-cell depletion, loss of CD4+ T cells in rectal tissues, and severe, fatal thrombocytopenia); and (vi) SHIV-2873Nip was mucosally transmissible and neutralization sensitive with a tier 1 profile.

SHIV-2873Nip carries the envelope gene of a pediatric HIV-C, the most prevalent strain worldwide. Although other SHIV strains (SHIVcEHN19, SHIVMa4, SHIV-MCGP1.3, and SHIV-XJO2170) encoding clade C envelopes have been created, they are either dual tropic (SHIV-MCGP1.3) (4), unable to replicate in RM PBMC (SHIVcEHN19) (5), or difficult to reisolate postadaptation (SHIVMa4 and SHIV-XJO2170) (37, 60). In contrast, SHIV-2873Nip was mucosally transmissible and was able to replicate vigorously in PBMC of all RM donors tested, indicating effective adaptation to the new host species. Only one other SHIV-C strain, SHIV-1157ipd3N4 (51), is exclusively R5 tropic and highly replication competent in RMs.

Genetic analysis of SHIV-2873Nip env showed a 5-aa deletion in the V4 region, a 3-aa deletion at the beginning of V5, and was still functional in the pseudotype virus assays in vitro and 14 point mutations throughout gp160. Interestingly, Env in the V4 region, a 3-aa deletion at the beginning of V5, extending into nef; (iii) SHIV-2873Ni and its passaged progeny contain extra NF-kB sites; (iv) SHIV-2873Nip retained exclusive R5 tropism; (v) SHIV-2873Nip showed signs of pathogenicity (memory T-cell depletion, loss of CD4+ T cells in rectal tissues, and severe, fatal thrombocytopenia); and (vi) SHIV-2873Nip was mucosally transmissible and neutralization sensitive with a tier 1 profile.

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Genetic analysis of SHIV-2873Nip env showed a 5-aa deletion in the V4 region, a 3-aa deletion at the beginning of V5, and 14 point mutations throughout gp160. Interestingly, Env was still functional in the pseudotype virus assays in vitro despite these deletions. In all monkeys infected mucosally, SHIV-2873Ni was cloned using the newly constructed molecular clone, SHIV-2873Ni, and the bi-

eral blood CD4+ T cells, primarily in mucosal tissues (3, 31, 57). We observed loss of memory CD4+CD29+ T cells in peripheral blood and depletion of CD4+ T cells in the gut tissues of all SHIV-2873Nip-infected RMs. This SHIV-C targets memory CD4+ T cells, whereas the acutely pathogenic, X4- or dual-tropic SHIVs that have been used frequently in vaccine efficacy studies in nonhuman primate models predominantly affect naive CD4+ T cells (16, 39) and induce precipitous drops in peripheral blood CD4+ T cells that are irreversible in most RMs (43, 45, 53). In contrast, SHIV-2873Nip does not induce acute, severe lymphocyte depletion, suggesting that this new R5-tropic SHIV-C exhibits biological characteristics that mimic HIV disease progression in humans. Another R5-tropic SHIV that carries an HIV clade B env, SHIVSF62P1, also induces gradual CD4+ T-cell loss and causes AIDS in some but not all RMs (15). Recently, Pahar et al. (40), using repeated low-dose vaginal SHIVSF62P1 challenges, observed control of viremia in most animals with modest depletion of the memory CD4+ T-cell subsets. In our repeated low-dose i.r. challenge approach, SHIV-2873Nip led to statistically significant depletion of CD4+ T cells in the gut.

SHIV-2873Nip induced fatal thrombocytopenia in one RM. Thrombocytopenia is a known complication of lentiviral infection and has been associated with all stages of HIV infection in humans (reviewed in reference 18) as well as SIV and SHIV infection in macaques (reviewed in references 17, 33, and 51). A relatively recent population-based study examined the association between AIDS and strokes; a strong link was found with both intracerebral hemorrhages and ischemic strokes (52). An earlier report described an association between thrombocytopenia and the development of intracerebral hemorrhages in patients with AIDS (42).

Our data indicate that SHIV-2873Nip is sensitive to neutralization by human NMAbs, polyclonal sera of SHIV-C-infected RM and HIV-C-infected humans, and a polyclonal high-titer anti-HIV Ig preparation (HIVIG) which had been generated from HIV clade B-infected individuals. SHIV-2873Nip was more neutralization sensitive than SHIV-1157ipd3N4. This is probably due to the fact that we reisolated SHIV-2873Nip approximately a year after RM RNi-9 became infected. In contrast, SHIV-1157ipd3N4 was generated from a virus reisolated after a significantly longer period of time from an infected RM that had progressed to AIDS at 2.7 years postinfection; SHIV-1157ipd3N4 was clearly a neutralization escape virus (51). According to an intriguing study, recently transmitted HIV-C isolates were surprisingly neutralization sensitive compared to donor virus strains (10, 24). Among discordant couples, newly infected individuals harbored more neutralization-sensitive viruses compared with the strains that predominated in their infected partners when tested against contemporaneous donor plasma, suggesting that a bottleneck effect during or shortly after sexual transmission favored neutralization-sensitive HIV-C quasispecies. The newly transmitted HIV-C strains had significantly fewer N-linked glycosylation sites and shorter variable loops than the strains that predominated in the infected source persons (10, 24).

Of note, SHIV-2873Nip was classified as a tier 1 virus based upon its susceptibility to neutralization by polyclonal sera collected from HIV-C-infected individuals, HIVIG, and NMAbs; this high neutralization sensitivity will be useful in the development of NAb response-based AIDS vaccine concepts. To date, the induction of sufficient NAb levels with extended breadth has been a major hurdle. We suggest that initial vaccine efficacy testing should make use of a tier 1 SHIV challenge virus in primate models. If protection is achieved, subsequent vaccine development steps could then use SHIV strains that are progressively more difficult to neutralize in primates. SHIV-2873Nip will be useful tool to evaluate vaccine candidates that seek to induce anti-HIV-C NAb responses, and as of today, it is the only SHIV-C tier 1 virus described.

Our in vivo data indicate that SHIV-2873Nip is a highly replication-competent R5-tropic SHIV-C. We have previously generated SHIV-1157ipd3N4, a tier 2 R5-tropic SHIV-C that is pathogenic and induces AIDS (51), although disease progression has been slow. Nevertheless, SHIV-1157ipd3N4 has been used successfully to assess the efficacy of a multigenic DNA prime/protein boost and a multigenic protein-only vac-
cine (44). The following parameters have been used as read-outs of vaccine efficacy: complete protection from systemic viral infection, as well as delay and lowering of peak viremia. We posit that the same strategy can be applied to challenge studies involving the tier 1 SHIV-2873Nip. In addition, protection from depletion of gut CD4+ lymphocytes could serve as a measurement of vaccine efficacy. Prolonged, prospective follow-up will reveal whether SHIV-2873Nip is more pathogenic than SHIV-1157ipd3N4.

In summary, the R5-tropic SHIV-2873Nip is a highly replication-competent, mucosally transmissible tier 1 SHIV-C that will be a useful tool to study viral pathogenesis, to assess the efficacy of immunogens targeting HIV-C Env, and to test vaccine candidates that seek to induce anti-HIV-C NAb responses.

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