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Non-Macrophage-Tropic Human Immunodeficiency Virus Type 1 R5 Envelopes Predominate in Blood, Lymph Nodes, and Semen: Implications for Transmission and Pathogenesis

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Human immunodeficiency virus type 1 (HIV-1) R5 isolates that predominantly use CCR5 as a coreceptor are frequently described as macrophage tropic. Here, we compare macrophage tropism conferred by HIV-1 R5 envelopes that were derived directly by PCR from patient tissue. This approach avoids potentially selective culture protocols used in virus isolation. Envelopes were amplified (i) from blood and semen of adult patients and (ii) from plasma of pediatric patients. The phenotypes of these envelopes were compared to those conferred by an extended panel of envelopes derived from brain and lymph node that we reported previously. Our results show that R5 envelopes vary by up to 1,000-fold in their capacity to confer infection of primary macrophages. Highly macrophage-tropic envelopes were predominate in brain but were infrequent in semen, blood, and lymph node samples. We also confirmed that the presence of N283 in the C2 CD4 binding site of gp120 is associated with HIV-1 envelopes from the brain but absent from macrophage-tropic envelopes amplified from blood and semen. Finally, we compared infection of macrophages, CD4⁺ T cells, and peripheral blood mononuclear cells (PBMCs) conferred by macrophage-tropic and non-macrophage-tropic envelopes in the context of full-length replication competent viral clones. Non-macrophage-tropic envelopes conferred low-level infection of macrophages yet infected CD4⁺ T cells and PBMCs as efficiently as highly macrophage-tropic brain envelopes. The lack of macrophage tropism for the majority of the envelopes amplified from lymph node, blood, and semen is striking and contrasts with the current consensus that R5 primary isolates are generally macrophage tropic. The extensive variation in R5 tropism reported here is likely to have an important impact on pathogenesis and on the capacity of HIV-1 to transmit.

Human immunodeficiency virus type 1 (HIV-1) requires interactions with CD4 and a coreceptor, usually CCR5 or CXCR4, to trigger fusion of viral and cellular membranes and entry into cells. CXCR4-using (X4) variants can be isolated from about 50% of AIDS patients and are associated with a more rapid loss of CD4⁺ T cells and faster disease progression (1, 8, 30, 36). CXCR4 is more widely expressed on different CD4⁺ T-cell populations compared to CCR5, and X4 viruses thus have a broader T-cell tropism (3, 26). Nevertheless, CD4 depletion and AIDS occur in patients from whom only CCR5-using viruses can be isolated (6, 10). Transmitted viruses are usually CCR5-using (R5) viruses, and individuals homozygous for a defective CCR5 gene ($\Delta 32$ CCR5) are substantially protected from infection (5).

HIV-1 isolates that predominantly use CCR5 as a corecep-

tor have been described as R5 or macrophage tropic (2). However, HIV-1 R5 viruses vary considerably in cell tropism properties (11, 18, 33). We reported that primary HIV-1 R5 isolates varied in their capacity to infect primary macrophage cultures by over 1,000-fold (33). We also first described a subset of HIV-1 R5 isolates that could infect CD4⁺ T-cell lines via trace amounts of CCR5 (11), and we recently described HIV-1 R5 envelopes from brain tissues of individuals with neurological complications that were highly fusigenic and tropic for macrophages (27). These latter envelopes are able to exploit low amounts of CD4 and/or CCR5 for infection and contrast emphatically with envelopes from immune tissue (lymph node) that failed to confer macrophage infection and required higher amounts of CD4 for infection. Since the capacity to exploit low levels of CD4 and/or CCR5 conferred a broader tropism for both T-cell lines and macrophages, we have tentatively termed these distinct R5 tropisms as narrow/R5 and broad/R5 (27).

The impact of diverse R5 tropism on transmission or pathogenesis is unknown. Several groups have reported that viral isolates from late in disease carry an enhanced macrophage tropism (16, 22, 38) perhaps similar to that conferred by highly

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TABLE 1. Details of patients studied

Patient	Age	Status	Disease stage ^c	CD4 count (cells/mm ³)	Neurological involvement	Sample(s)
P3	Adult	Homosexual	B2	330	No	Blood, semen
P31	Adult	Homosexual	C3	130	No	Blood, semen
P43	Adult	Homosexual	A1	620	No	Blood, semen
P-1206	Neonate	MTCT ^a	C2	1,119	No	Plasma
			C3	218	Yes	Plasma
P-1114	Neonate	Sexual	A2	660	No	Plasma
			A2	1,108	No	Plasma
			C3	46	Yes	Plasma
P-1031	Neonate	MTCT	N1	2,147	No	Plasma
			C2	250	Yes	Plasma, CSF
NA118	Adult	IVDU ^b	C3	80	Yes	Frontal lobe, lymph node
NA420	Adult	Heterosexual	C3	12	Yes	Frontal lobe, lymph node
NA20	Adult	Hemophilic	C3	4	Yes	Frontal lobe, lymph node
NA176	Adult	IVDU	C3	20	Yes	Frontal lobe, lymph node
NA353	Adult	IVDU	C3	2	Yes	Frontal lobe

^a MTCT, mother-to-child transmission.

^b IVDU, intravenous drug user.

^c CDC disease stage classification.

macrophage-tropic brain envelopes (27). Most studies of HIV tropism have been carried out using viruses that were isolated by culture in peripheral blood mononuclear cells (PBMCs). This procedure is likely to be highly selective, favoring variants that replicate faster and efficiently infect T cells.

Here, we examine macrophage tropism of 33 envelopes amplified from semen and blood of HIV-1⁺ adults and from the plasma and cerebral spinal fluid (CSF) of HIV-1⁺ pediatric patients. These envelopes were amplified by PCR directly from patient material, thus avoiding any viral replication that could confer mutation. The envelopes amplified were compared with an extended panel of envelopes from brain and lymph node samples that were reported previously (27). Brain tissue is usually protected by the blood brain barrier and contains low concentrations of neutralizing antibodies. The role of this immunoprivileged environment in the evolution of highly macrophage-tropic variants is not known. In addition, HIV in semen is believed to originate from both immune and nonimmune tissue, including seminal vesicles in the testes, the prostate, and the urethra (19), as well as from immune cells that infiltrate from inflamed tissue (41). Furthermore, the testes are protected by the testis blood barrier (43) and may also represent an immunoprivileged environment. Finally, memory T cells (the main T-cell target for R5 viruses) comprise a smaller percentage of total T cells in neonates and infants compared to adults (31, 34). Neonates may thus also represent a distinct environment for HIV replication compared to adults. Each of these different environments may influence the evolution of HIV envelope phenotypes.

Here, we show that in contrast to the brain, macrophage-tropic R5 envelopes are infrequent in semen, blood, and lymph nodes.

MATERIALS AND METHODS

Cells. HeLa TZM-BL cells were provided by the NIH AIDS Research and Reference Reagent Program. HeLa cell clones were provided by Navid Madani (Dana Farber Cancer Institute, Boston, Mass.) and David Kabat (Oregon Health and Science University, Portland) (29). NP2/CD4/CCR5 and NP2/CD4/CCR3 (35) were provided by Hiroo Hoshino at Gunma University Graduate School of

Medicine, Maebashi, Japan. Primary macrophages, CD4⁺ T cells, and PBMCs are described below.

Patient samples. HIV-1 envelopes were amplified as part of three separate studies from (i) blood and semen of adult patients, (ii) plasma and CSF of pediatric patients, and (iii) brains and lymph nodes of adult patients. Table 1 summarizes the patient material that was used in this study. Semen- and blood-derived envelopes were from three adults at different disease stages (9). Pediatric-derived envelopes were from plasma taken from early and late disease stages of three patients. Brain- and lymph node-derived envelopes were from five AIDS patients with neurological complications and were described previously (27).

PCR amplification of envelopes. Envelope genes were amplified from patient tissue samples by nested PCR using the Expand High-Fidelity PCR system (Roche Inc.) or KOD XL DNA polymerase (Novagen Inc.). A nested PCR protocol was used as described by Gao et al. (15). Outer primers were Env A (GGC TTA GGC ATC TCC TAT GGC AGG AAG AA) and Env N (CTG CCA ATC AGG GAA GTA GCC TTG TGT), and inner primers were Env B (AGA AAG AGC AGA AGA CAG TGG CAA TGA) and Env M (TAG CCC TTC CAG TCC CCC CTT TTC TTT TA). Conditions for both rounds of PCR were as follows: first round, 94°C for 45 s, 50°C for 45 s, and 72°C for 4 min (20 cycles); second round, 94°C for 45 s, 50°C for 45 s, and 72°C for 4 min 15 s (15 cycles).

Brain and lymph node envelopes were amplified from proviral DNA from autopsy tissue samples as described previously (27). Envelopes from P3, P31 and P43 were amplified by limiting dilution from proviral DNA from semen and blood cells (9). Similar procedures were followed to amplify envelopes from pediatric HIV-1 RNA in plasma following synthesis of envelope cDNA using SuperScript reverse transcriptase (RT; Invitrogen Inc.). All envelopes were amplified using the Expand High-Fidelity PCR system (Roche Inc.) except for C98-28, C98-67, and S94csf-11 envelopes, which were amplified with KOD XL DNA polymerase (Novagen Inc.). Most PCR products were first cloned into pCR-XL-TOPO (Invitrogen Inc.) and then into the pSVIIIenv expression vector via conserved KpnI sites. For several envelopes, PCR products were digested with KpnI and ligated directly into pSVIIIenv. Two P43 envelopes (378.2 and 380.1) contained only one of the KpnI sites and were subjected to limited PCR to reintroduce the second site.

Phylogenetic analysis. All V1 to V3 gp120 env nucleotide sequences were assembled and aligned using CHROMAS W (37). Sequences were adjusted manually. All positions with an alignment gap of one or more nucleotides were excluded. The tree construction was performed from the same distance matrices with the neighbor-joining algorithm and Kimura two-parameter model (transition/transversion ratio, 2.0) to estimate the nucleotide distances. Bootstrap analysis on 1,000 replicates was used to assess the robustness of the tree. The phylogenetic tree was generated using the program MEGA version 3 (20). The NL4.3 reference sequence was used as an outgroup.

Production of envelope-positive pseudotype viruses. The env-negative pNL4.3 construct and pSVIIIenv expression vector were described previously (27). Pseudotype viruses carrying each patient envelope were produced by cotransfection of Env⁺ pSVIIIenv with Env⁻ pNL4.3 into 293T cells using calcium phos-

phate. Cell supernatants carrying progeny pseudotype virions were harvested 48 h after transfection, clarified ($1,000 \times g$ for 10 min), aliquoted, and stored at -152°C .

Replication-competent clones. NA420 B33 and LN85, NA118 LN33, and NA20 B59 envelopes were subcloned into pNL4.3 using a BbsI site at the start of the envelope and an XhoI site downstream from the 3' end of the envelope. LN33 lacked an XhoI site and was cloned via a proximal BlnI site. A version of NA420 LN85 envelope that contained B33 gp41 sequences was used (27). Replication competent clones of YU2 (23) and JR-CSF (18) were obtained from the NIH AIDS Research and Reference Reagent Program.

Preparation of macrophage cultures. Elutriated monocytes (17) provided by the University of Massachusetts Center for AIDS Research Elutriation Core were treated with macrophage colony-stimulating factor (R&D Systems) and cultured for 5 to 7 days before use for virus infections. Alternatively, macrophages were prepared from blood monocytes as described previously (32). Briefly, fresh blood (from volunteer donors) was diluted 1:1 in RPMI 1640 medium and carefully layered onto 15 ml of Ficoll Hypaque (Pharmacia) in a 50-ml Falcon tube and centrifuged at $350 \times g$ for 30 min at room temperature. White cells harvested from the interphase between Ficoll and plasma and medium were washed in RPMI 1640 and resuspended. Cells were then plated onto a 15-cm-diameter bacterial petri dish with 10^8 cells in 20 ml. Cells were incubated for 3 h at 37°C . The adherent cells were then gently washed twice with fresh RPMI 1640 5% human plasma, before 15 ml RPMI 1640 containing 10% human plasma was added and cells were incubated at 37°C overnight. The following day the plates were washed again to remove nonadherent cells. Then fresh media was added. Cells were allowed to differentiate into macrophages over the next 5 to 7 days. The day prior to infection, the macrophages were washed three times in versene and incubated at 37°C for 10 minutes to loosen cell attachments. Macrophages were then gently scraped off and resuspended in RPMI 1640 containing 10% human plasma, counted, and seeded into 48-well tissue culture trays at 1.25×10^5 cells per well.

Preparation of PBMCs and CD4⁺ T cells. PBMCs were prepared from buffy coats (Research Blood Components LLC.). Briefly, buffy coats were diluted 1:5 in RPMI 1640 and centrifuged onto Ficoll Paque. Leukocytes were removed from the interface, washed twice in RPMI 1640, and resuspended at 1×10^6 cells/ml in RPMI 1640 containing 5 $\mu\text{g/ml}$ phytohemagglutinin, 10% fetal bovine serum, and gentamicin. After two days in culture, cells were resuspended in fresh RPMI 1640 containing 10 units/ml interleukin-2, 10% fetal bovine serum, and gentamicin. Cells were infected with HIV-1 between 5 and 7 days from the initiation of culture.

CD4⁺ T cells were purified by negative selection from PBMCs after 5 to 7 days of culture as described above. Briefly, cells were treated with a cocktail of antibodies (StemCell Technologies Inc.) and magnetic particles that bind all cells except CD4⁺ T cells. All cells except CD4⁺ T cells were removed using an EasySep magnet (StemCell Technologies Inc.). Enriched CD4⁺ T cells (generally >98% pure) were then seeded into 96-well culture trays at 2×10^5 cells per well for immediate infection.

Coreceptor use of HIV-1 envelopes. Coreceptors used by each envelope were evaluated in cell-cell fusion assays. Cell-cell fusion was tested by cocultivating appropriate test cells with 293T cells cotransfected with env⁺ pSVIIIenv and Env⁻ pNL4.3. Cell fusion via CCR2b, CCR3, CCR5, CX3CR1, CXCR6, and GPR15 was tested using GHOST cells. Fusion via CCR3, CCR5, CCR8, GPR1, APJ, and CXCR4 was tested using NP2/CD4 cells. Coreceptor⁺ NP2/CD4 or GHOST cells were seeded at 8×10^4 cells per well in 48-well culture dishes and incubated overnight. 293T cells were transfected as described above and also incubated overnight. Each transfected 293T cell culture was trypsinized, and 50 μl of cells (2×10^5 cells/ml) was cocultivated with coreceptor-positive NP2/CD4 or GHOST cells. After 6 h of incubation, cocultivations with coreceptor-positive NP2/CD4 or GHOST cells were washed in phosphate-buffered saline (PBS) and fixed for 10 min in methanol containing 1% methylene blue, 0.25% basic fuchsin. Fixed cells were destained in PBS and examined by light microscopy for the presence of syncytia. We showed previously that cell-cell fusion assays (although less quantitative) accurately predict coreceptors used by envelope-positive pseudoviruses (27).

Infection of HeLa/CD4/CCR5 cells and macrophages. All envelope-positive pseudotyped viruses were titrated onto HeLa TZM-BL cells and primary macrophage cultures. HeLa TZM-BL cells are derived from the JC53 clone of HeLa/CD4/CCR5 cells (29), which express high levels of both CD4 and CCR5. HeLa TZM-BL cells contain both β -galactosidase and luciferase reporter genes (40). Infection of HeLa TZM-BL cells was assessed 2 days after infection by fixing in cold (4°C) 0.5% glutaraldehyde in PBS and then adding 5-bromo-4-chloro-3-indolyl- β -galactopyranoside (X-Gal) substrate (Fisher Scientific Company LLC) at 0.5 mg/ml in PBS containing 3 mM potassium ferrocyanide, 3 mM

potassium ferricyanide, 1 mM magnesium chloride. Infected cells stained blue after about 1 hour of incubation at room temperature.

HeLa cell clones expressing different levels of CD4 and CCR5 were as follows: JC53, JC37, and JC10 were reported to express 1.3×10^5 , 1.5×10^4 , and 2×10^3 CCR5 molecules on the cell surface and were cloned from HI-J, which expressed 4×10^5 CD4 molecules (29). RC49, RC23, and RC33 were reported to express 8.5×10^4 , 8.7×10^3 , and 3.2×10^3 CCR5 molecules and were cloned from HI-R that expressed 10^4 CD4 molecules (29). These cells were frozen and used over a period of 10 passages where receptor levels have been confirmed as high, medium, and low by flow cytometry. HeLa clones were treated with DEAE dextran and infected as described for HeLa TZM-BL cells. Cells were fixed after 3 days of culture and immunostained for p24 as previously described (7). Briefly, cells were washed once in PBS and then fixed in cold (-20°C) methanol/acetone at a ratio of 1:1 for 10 min at room temperature. Fixed cell layers were then washed once with PBS and once with PBS containing 1% fetal calf serum (FCS) before adding anti-HIV-1 p24 monoclonal antibodies (MAbs 38:96K and EF7; Centre for AIDS Reagents, United Kingdom). These MAbs were added as hybridoma cell culture fluid at a 1:1 ratio diluted 1 in 40 in PBS-1% FCS and incubated at room temperature for at least 1 hour. Cells were washed once in PBS-1% FCS before incubating for at least 1 h at room temperature with anti-mouse F(ab')₂ fragments conjugated to β -galactosidase (Southern Biotechnology Associates, Inc.) at a dilution of 1 in 400 in PBS-1% FCS to detect the first antibody layer. Cells were then washed once with PBS-1% FCS and twice with PBS. Infected cells stained blue on addition of X-Gal substrate as described above.

Macrophage infection was evaluated by fixing infected macrophages in methanol-acetone (1:1) 7 days postinfection and immunostaining for intracellular p24 antigen as described above.

The pseudotype viruses prepared are competent only for a single round of replication. Thus, individual or groups of recently divided, blue-stained cells were regarded as foci of infection. For replication-competent viruses, well-separated areas of p24 staining were regarded as foci. Virus infectivity for HeLa cells and macrophages was estimated as the focus-forming units per milliliter (FFU/ml).

Infection of CD4⁺ T-cell and PBMC cultures. Infectivity titers for CD4⁺ T cells and PBMCs were estimated using a 50% tissue culture infective dose (TCID₅₀) approach. Briefly, CD4⁺ T cells and PBMCs cultured for 5 to 7 days (as described above) were seeded into wells of a 96-well flat-bottomed tissue culture dish at 2×10^5 cells in 50 μl of growth medium. Fifty-microliter aliquots of replication-competent virus were added as serial dilutions in replicates of six. After 7 days of culture, supernatant was harvested and analyzed for RT activity. TCID₅₀s were calculated using the Karber method (24).

RT enzyme-linked immunosorbent assay. The Lenti-RT activity assay (Cavidi Tech, Uppsala, Sweden) was used for RT enzyme-linked immunosorbent assay of supernatants obtained from infected cultures according to the manufacturer's instructions.

RESULTS

Sequence analysis of envelope genes. Envelopes were sequenced from V1 to V3. Figure 1 shows a phylogenetic tree of all envelopes used in this study. Envelopes clustered into groups representing the different patients.

Coreceptor use of envelopes. Coreceptor use was evaluated using cell-cell fusion assays. None of the envelopes reported here used CXCR4. The vast majority of envelopes used CCR3 and CCR5 and efficiently induced cell-cell fusion in NP2/CD4/CCR3 and NP2/CD4/CCR5 cells. Several pediatric envelopes (C98-67, J92-18, and J92-39) also weakly used CCR8. As reported previously, NA420 brain envelopes (B13, B33, and B42) were CCR5 specific. NA20 B59 used a range of coreceptors (27), while NA20 B503 used CCR8 in addition to CCR3 and CCR5.

Macrophage tropism of envelopes. Envelope-positive virions harvested 48 h after transfection of 293T cells were titrated on HeLa TZM-BL cells, which express high levels of CD4 and CCR5 (29), and on at least two primary macrophage cultures derived from different donors. Figure 2 shows macrophage infectivity as a percentage of the titer recorded on HeLa TZM-BL cells. Thus, the higher the percentage, the higher the infectivity or tropism for macrophages. Highly macrophage-

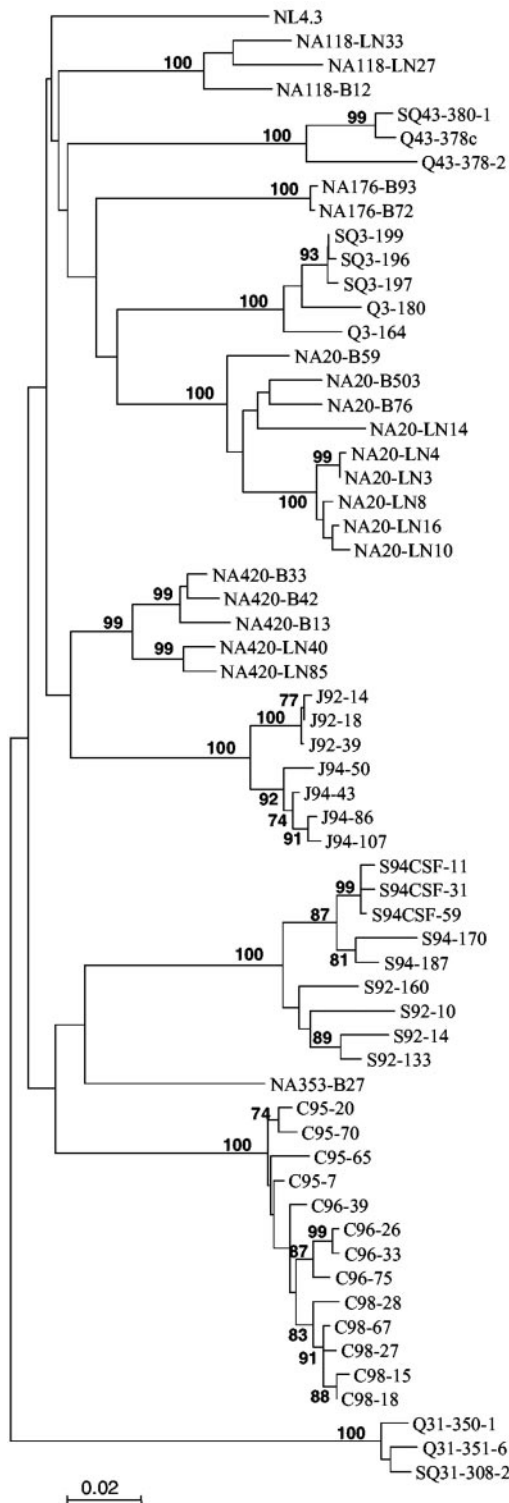


FIG. 1. Phylogenetic analysis of the V1 to V3 sequences of envelopes investigated. V1 to V3 gp120 Env sequences were used to construct the phylogenetic tree by the neighbor-joining method. The sequence of the NL4.3 virus was used as an outgroup. The percentage of bootstrap repetitions (out of 1,000) in which the nucleotide sequences grouped together is represented as a number at the branch node, but only the bootstrap values of >74% are shown. The genetic distance was 2%. Envelopes from pediatric patients P-1206, P-1114, and P-1031 are designated by the letters S, C, and J, respectively.

tropic envelopes were rare in blood, semen, and lymph nodes of adults. In contrast and as we have previously reported, macrophage-tropic envelopes were predominant in adult brain tissue. Furthermore, highly macrophage-tropic envelopes were infrequent in the three pediatric patients. Table 2 shows the fraction of macrophage-tropic envelopes detected for each site tested.

Macrophage tropism correlates with the capacity to exploit low levels of CD4 for infection. The effects of different cell surface concentrations of CD4 and CCR5 on infection were tested using a panel of HeLa cell clones that expressed high or low levels of CD4 and high, medium, or low levels of CCR5 (29). Using these HeLa cell clones, we previously reported that macrophage tropism correlated with the capacity to infect cells via low levels of CD4 (27). The same correlation was observed for the 10 adult blood and semen envelopes studied here (Fig. 2 and 3A). Thus, SQ43 380.1, which infected macrophages, also infected HeLa/CD4/CCR5 cells via low levels of CD4. In contrast, other non-macrophage-tropic blood and semen envelopes inefficiently infected HeLa/CD4/CCR5 cells via low levels of CD4.

Envelopes from pediatric plasma that infected macrophages also infected HeLa/CD4/CCR5 cells with low CD4 more efficiently than most other envelopes (Fig. 3B). One envelope derived from the CSF of an infected infant with neurological complications (S94-CSF-11) was highly macrophage tropic and conferred high infection of HeLa/CD4/CCR5 via low levels of CD4. The one exception was a non-macrophage-tropic envelope (S92-14) from the plasma of a pediatric patient that conferred relatively efficient infection of HeLa/CD4/CCR5 cells via low CD4. The capacities of brain and lymph node envelopes to infect the different HeLa cell clones were reported previously (27). Figure 3C shows an extended version of the 2004 data for comparison. Thus, macrophage-tropic R5 envelopes from brain conferred infection of HeLa cells via low levels of CD4, while lymph node-derived envelopes conferred inefficient infection at best.

Infection of primary CD4⁺ T-cell and PBMC cultures. Experiments described above used single-round envelope-positive pseudovirions and used HeLa TZM-BL cells as a standard permissive cell line, which expresses high amounts of CD4 and CCR5. We next tested whether the differences in macrophage tropism were maintained in the context of replication-competent viruses that carried brain- and lymph node-derived envelopes, as well as control macrophage-tropic envelope YU2 and non-macrophage-tropic envelope JR-CSF. We also evaluated whether the same replication-competent clones could replicate in cultures of primary CD4⁺ T cells or PBMCs. Replication-competent clones were titrated on HeLa TZM-BL cells, primary macrophages, CD4⁺ T cells, and PBMCs. Infectivity titers for HeLa TZM-BL cells and macrophages were determined by evaluating FFU, while infectivity for CD4⁺ T cells and PBMCs was estimated by TCID₅₀ analysis of RT data. Figure 4A shows that all envelope-positive viruses replicated to high titer in HeLa TZM-BL and CD4⁺ T cells and PBMCs. In contrast, non-macrophage-tropic envelopes (NA420 LN85, NA118 LN33, and JR-CSF) conferred about 100- to 1,000-fold lower infectivities for primary macrophages compared to highly macrophage-tropic envelopes (NA420 B33, NA20 B59, and YU2). Although infectivities for T cells and PBMCs are mea-

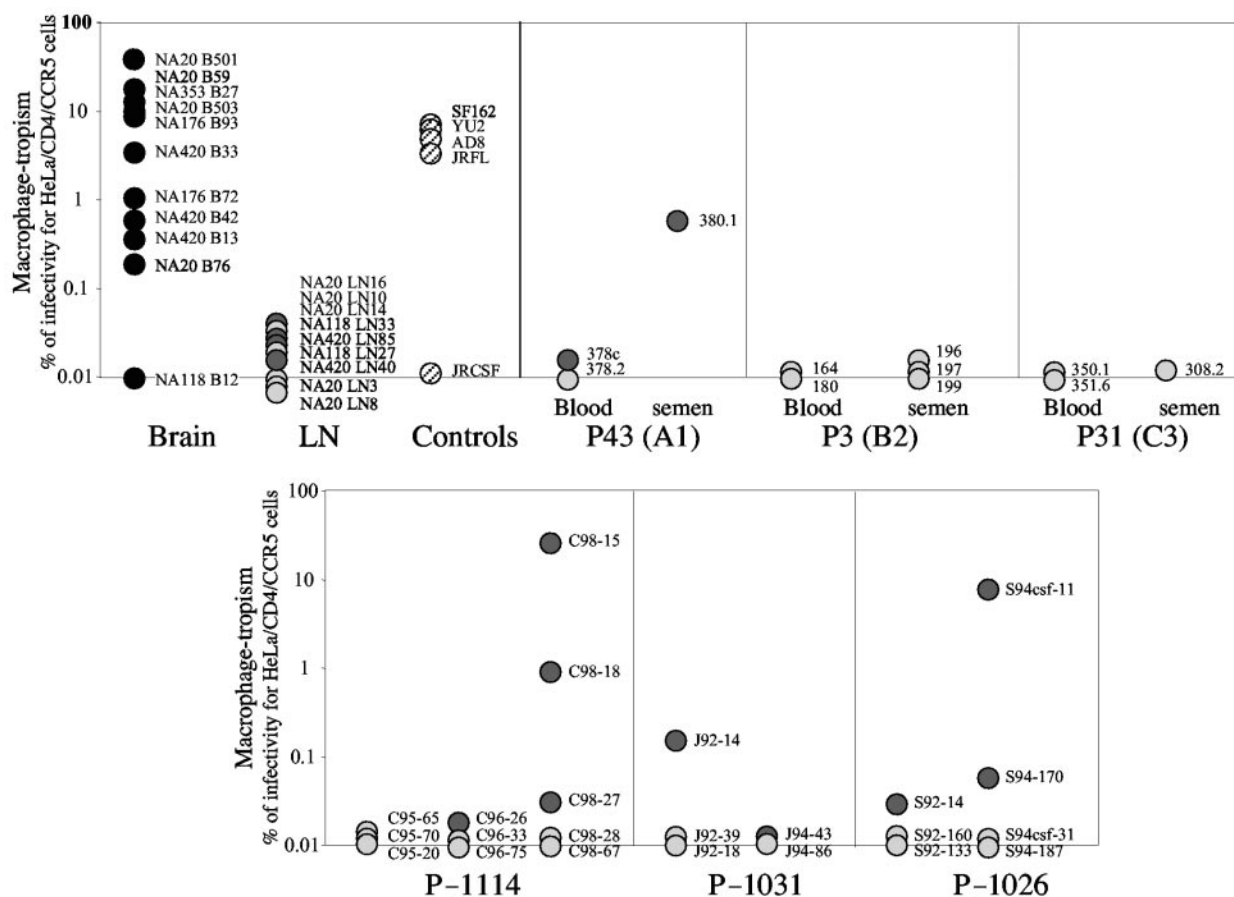


FIG. 2. Macrophage tropism of HIV-1 R5 envelopes amplified from patient tissues. Amplified envelopes were cloned into pSVIIIenv and expressed on HIV-1 particles. Infectivity was titrated on HeLa TZM-BL cells (HeLa/CD4/CCR5; JC53 clone [29]) and on primary macrophages. Infectivity was assessed by immunostaining in situ and estimating the number of infected cells as described previously (27). Macrophage infectivity is plotted as a percentage of that recorded on HeLa TZM-BL cells. Light symbols represent envelopes that failed to confer any macrophage infectivity, i.e., macrophage infectivity was less than the value shown.

TABLE 2. Macrophage tropism and presence of N283 in HIV-1 R5 envelopes^a

Study group	Source	No. of patients	Fraction of macrophage-tropic envelopes ^d	Frequency of N283 in C2 CD4 binding site
Adult	Brain, proviral	5	10/11	8/11
	Lymph node, proviral	3	0/9	1/9
	Blood, proviral	3	0/6	0/6
	Semen, proviral	3	1/5	0/5
Pediatric	Plasma, RNA	3	4/23	0/23
Controls	Macrophage tropic ^b		4/4	1/4
	Non-macrophage tropic ^c		0/1	0/1

^a N283 was present in the C2 CD4 binding site that lies just upstream of the V3 loop, for which the HXBc2 sequence is **TDNAKTHIVQLNTSVEINCTRPNNN** *TRKRIRIQRGPGRFVVTIGKIGNMRQ*AHC. T283 is in bold, while residues that contact CD4 are underlined. The V3 loop is in italics.

^b Envelopes SF162, JRFL, AD8, and YU2.

^c Envelope JR-CSF.

^d Fraction of envelopes that conferred macrophage infectivity of >1% of that recorded for SF162.

sured differently than for macrophages and HeLa TZM-BL cells, it is valuable to compare ratios of infectivity titers for macrophages with those recorded on PBMCs and CD4⁺ T cells as shown in Fig. 4B. Whether macrophage infectivity is compared to infectivity recorded on HeLa TZM-BL cells, PBMCs, or CD4⁺ T cells, the ratios conferred by non-macrophage-tropic JR-CSF, LN33, and LN85 are substantially lower (100- to 1,000-fold) than those conferred by macrophage-tropic envelopes YU2, B59, and B33. We also compared infectivity for macrophages, PBMCs, and CD4⁺ T cells as ratios of infectivity recorded on HeLa TZM-BL cells (Fig. 4C). While mac/TZM-BL ratios for non-macrophage-tropic envelopes were considerably lower than those for macrophage-tropic envelopes, PBMC/TZM-BL and CD4⁺ T-cell/TZM-BL ratios did not substantially differ between the tropism groups. These results confirm the extensive variation in macrophage tropism but indicate that tropism for CD4⁺ T cells and PBMCs is similar for highly macrophage-tropic and non-macrophage-tropic envelopes. Figure 4 also shows that the highly macrophage-tropic and non-macrophage-tropic phenotypes are maintained in the context of replication-competent viruses and are

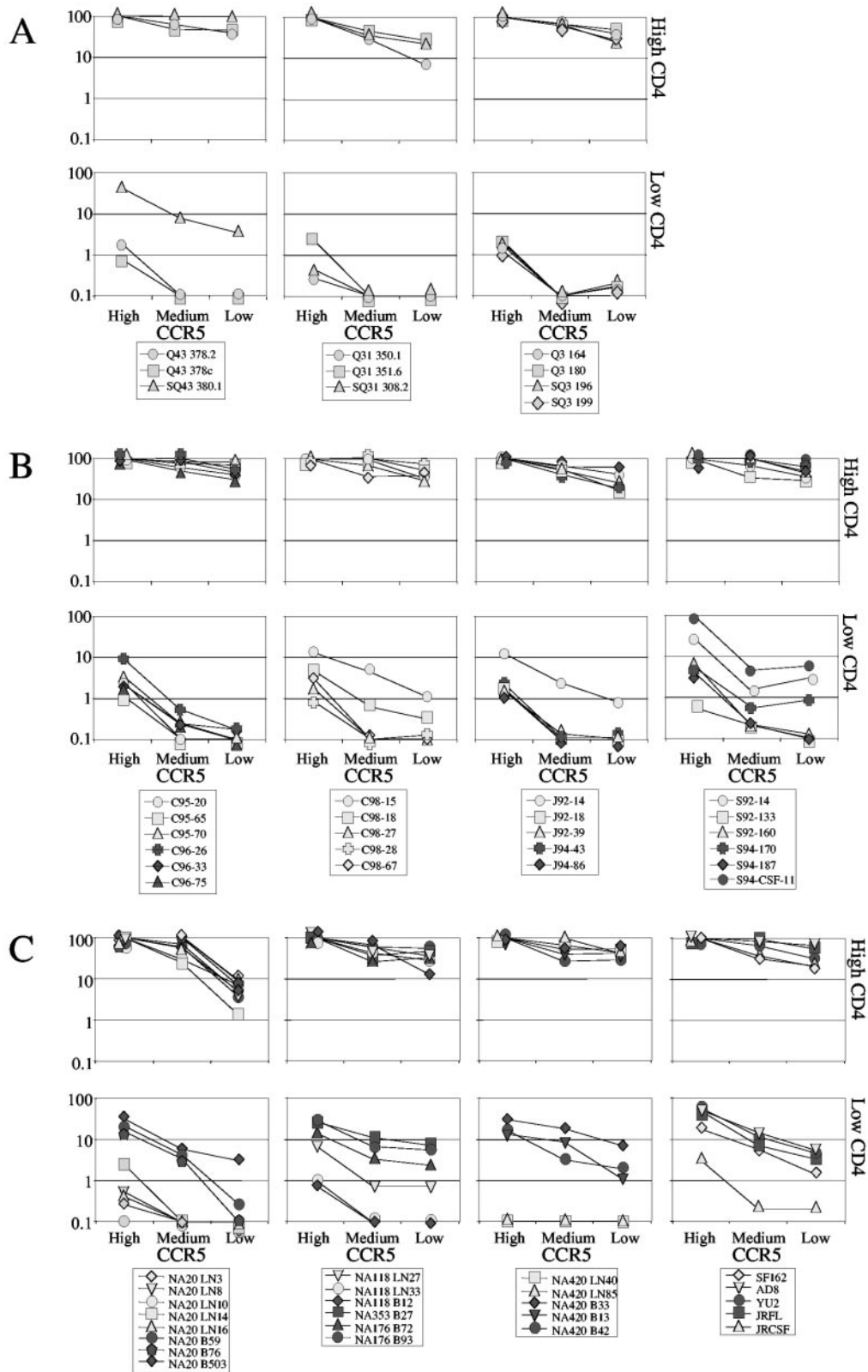


FIG. 3. Macrophage-tropic envelopes require less CD4 for infection. HeLa cell clones expressing different CD4 and CCR5 levels (29) were infected with Env⁺ pseudoviruses as described in Materials and Methods. After 72 h, cells were fixed and immunostained for p24 antigen, and foci of infection were counted. Infectivities for Env⁺ pseudotypes prepared with envelopes amplified from adult blood and semen (A) and pediatric plasma (B) are shown. Envelopes that conferred infection of macrophages (SQ43 380.1, C98-15, C98-18, J92-14, and S94-CSF-11) also conferred infection of HeLa cells expressing low levels of CD4. (C) Infectivities of an extended panel of brain and lymph node envelopes that were previously reported (27) are shown for comparison. Envelopes from pediatric patients P-1206, P-1114, and P-1031 are designated by the letters S, C, and J, respectively.

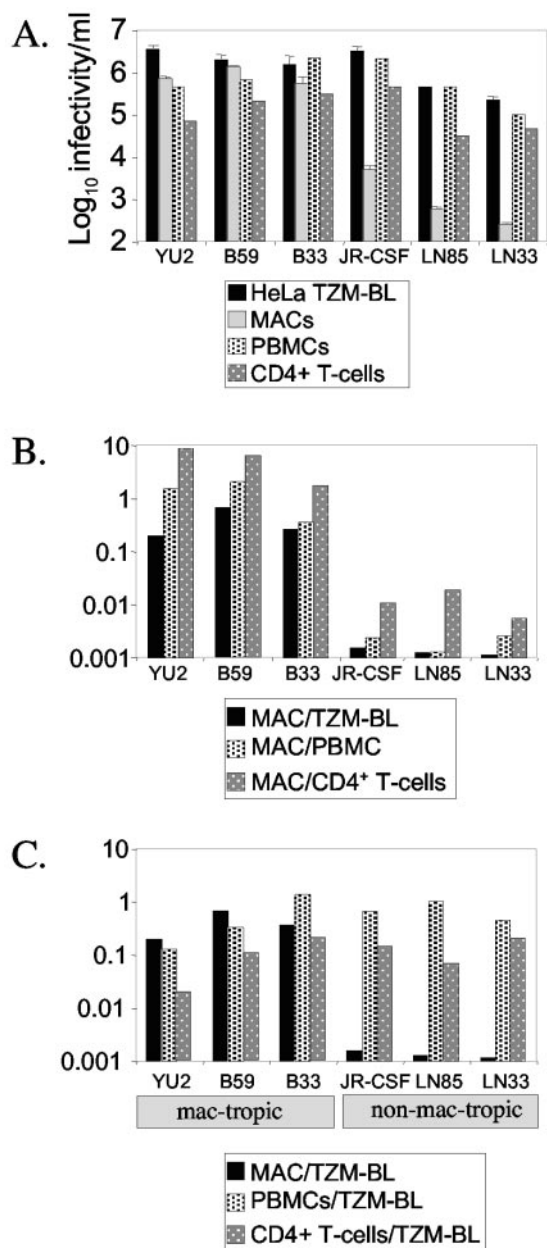


FIG. 4. Infectivities of replication-competent viruses carrying highly macrophage-tropic and non-macrophage-tropic envelopes evaluated on HeLa TzM-BL cells, primary macrophages, PBMCs, and CD4⁺ T cells. A. Infectivity titers of replication-competent viruses carrying macrophage-tropic (YU2, NA20 B59, and NA420 B33) or non-macrophage-tropic (JR-CSF, NA420 LN85, and NA118 LN33) envelopes. Infectivities for HeLa TzM-BL cells and macrophages were estimated by FFU assays, and the results shown represent averaged FFU counts for duplicate wells from one of several experiments with similar results. Infectivities for PBMCs and CD4⁺ T cells were estimated by TCID₅₀ assay, using replicates of six wells, and are representative of at least two experiments. B. Ratios of infectivity for macrophages compared to HeLa TzM-BL cells, PBMCs, and CD4⁺ T cells. C. Ratios of infectivity for macrophages, PBMCs, and CD4⁺ T cells compared to HeLa TzM-BL cells.

not an artifactual result of the use of single-round pseudovirion assays.

Role of N283 in the C2 CD4 binding site. The C2 CD4 binding site lies just upstream from the V3 loop in the envelope. The HXBc2 amino acid sequence spanning this region is shown in Table 2, footnote *a*. Residues 279D, 280N, 281A, and 283T were shown to directly contact CD4 in the crystal structure of gp120 and the CD4 complex (21). An asparagine residue at position 283 in the C2 CD4 binding site has been reported to occur more frequently in envelopes present in the brain and to confer a more efficient interaction between gp120 and CD4 (13, 14). Table 2 confirms that N283 was frequent among the brain-derived macrophage-tropic envelopes examined here. N283 was absent from pediatric plasma and from adult semen- and blood-derived envelopes, including those that conferred macrophage infection. Only one lymph node envelope (NA20 LN14) contained N283. However, while LN14 clustered phylogenetically with NA20 envelopes from the brain (Fig. 1), it conferred a non-macrophage-tropic phenotype similar to other lymph node-derived envelopes. These observations confirm the prevalence of N283 in macrophage-tropic envelopes in brain but indicate that it is not the only determinant of macrophage tropism.

DISCUSSION

Our results show that R5 envelopes vary by up to 1,000-fold in their capacity to confer infection of blood-derived primary macrophages. Thus, highly macrophage-tropic envelopes were found frequently in the brain but infrequently in semen, blood, and lymph node samples. The lack of macrophage tropism for the majority of the envelopes amplified from lymph node, blood, and semen is striking and contrasts with a widely held view that R5 primary isolates are generally macrophage tropic (2).

In this study, PCR-amplified envelopes were coexpressed with an Env⁻ pNL4.3 construct in 293T cells to produce pseudovirions capable of just a single round of infection. A concern is that such pseudovirions may not represent replication-competent viruses present in vivo and may underestimate macrophage infection. In control experiments, we have shown that different patient envelopes assemble onto pseudovirions with slightly different efficiencies. However, these different assembly efficiencies did not correlate with the extent of macrophage infectivity (data not shown). Figure 4 shows that full-length infectious chimeric clones (based on pNL4.3) that carry different brain- and lymph node-derived envelopes conferred the same macrophage-tropic and non-macrophage-tropic phenotypes as the corresponding envelope-positive pseudovirions. Virus produced from 293T cells following transfection of these infectious clones conferred at least 50-fold higher infectivity than pseudovirions. Nevertheless, ratios of infectivity for macrophages compared to HeLa TzM-BL cells were up to 1,000-fold lower for non-macrophage-tropic envelopes compared to highly macrophage-tropic envelopes from the brain. Thus, the experiments shown in Fig. 4 strongly support the relevance of the data generated using envelope-positive pseudovirions but show that the non-macrophage-tropic envelopes are not negative for macrophage infection, just very inefficient. It was also possible that virions budding from 293T cells may lack the necessary adhesion molecules required for macrophage infec-

tion. However, Bounou et al. reported that virions produced from 293T cells that express ICAM-1 attached to primary macrophages with similar efficiencies to virions lacking ICAM-1 (4). Bounou et al. concluded that attachment was conferred by CD4-independent and adhesion molecule-independent mechanisms that likely involve envelope sugars and the macrophage mannose receptor. This is supported by Nguyen and Hildreth, who showed that the macrophage mannose receptor was the predominant mode of attachment to macrophages by HIV particles (25).

We also evaluated whether brain and lymph node envelopes conferred infection of primary CD4⁺ T-cell and PBMC cultures. Figure 4 demonstrates that replication-competent clones carrying non-macrophage-tropic lymph node envelopes (NA420 LN85 and NA118 LN33) conferred high levels of infectivity for both CD4⁺ T cells and PBMCs. These results confirm that non-macrophage-tropic envelopes amplified from immune tissue are fully competent for replication in primary CD4⁺ T cells and are not compromised for infection of primary cell types generally.

The determinants of the distinct R5 tropisms are not yet fully defined. The presence of N283 is likely to play a major role in the macrophage tropism of envelopes in the brain. Dunfee et al. (13) have modeled N283 onto the gp120 structure (21) and shown that N283 more readily forms a hydrogen bond with Q40 on CD4 and presumably helps to stabilize the gp120-CD4 interaction. Regardless, macrophage-tropic envelopes amplified from pediatric patients and from adult semen did not carry N283, indicating that this residue was not the sole determinant for macrophage infection. In addition, NA20 LN14 did carry N283 and clustered phylogenetically with NA20 brain envelopes yet failed to infect macrophages. Thus, the impact of N283 on macrophage infection can be overcome by other determinants in envelope. For example, Walter et al. reported that sequences within the V1V2 loops modulated macrophage infectivity conferred by chimeric envelopes derived from macrophage-tropic R5 BAL and non-macrophage-tropic X4 NL4.3 HIV-1 isolates (39). The V1V2 loops sit over gp120 sites involved in binding coreceptors (42) but may also occlude the CD4 binding site (28). It is also possible that glycosylation groups (12), other variable loops, or other structural alterations may occlude the CD4 binding site. These protective strategies may reduce the efficiency of gp120-CD4 binding even in the presence of N283.

Neutralizing antibodies that target the CD4 binding site on gp120 may drive evolution of envelope conformations that protect this site. Since the brain usually contains lower levels of neutralizing antibodies compared to immune tissue, this environment may allow the evolution of variants that interact efficiently with CD4 and can replicate in macrophage lineage cells resident in the brain. Viral replication in PBMC cultures (in the absence of neutralizing antibodies) during isolation may also select for variants proficient for macrophage infection; however, they may not be representative of HIV-1 quasispecies in vivo.

In this study, we investigated envelopes from multiple samples and patients rather than multiple envelopes from a few samples. To this end, we have studied 53 envelopes derived from 22 samples of 11 patients. For most samples, we were able to study only one to three envelope clones and therefore have

not extensively sampled the viral population present. It is therefore possible that highly macrophage-tropic variants are present at lower frequencies outside the brain, and this will require a more intensive investigation to evaluate.

The remarkable variation in R5 tropism shown here is likely to have a profound impact on pathogenesis, as also suggested by Gray et al. (16), and on the efficiency of transmission, depending on the importance of macrophages or cells that express low amounts of CD4 as targets for infection in the new host.

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